IDENTIFICATION OF LAWSONIA INTRACELLULARIS PROTEINS RECOGNIZED BY NEUTRALIZING ANTIBODIES AND USE OF THESE PROTEINS TO DESIGN A SUBUNIT VACCINE

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ABSTRACT

Lawsonia intracellularis is an obligate intracellular microorganism which causes diseases known as porcine ileitis or porcine proliferative enteropathy. Due to its obligate intracellular nature, characterization of *L. intracellularis* antigens and proteins involved in host-pathogen interaction and immune recognition have been difficult to accomplish using conventional microbiological techniques. The overall goal of this thesis is to identify, characterize and evaluate *L. intracellularis* bacterial proteins as neutralizing antibody targets which may then be suitable candidates for subunit vaccine development. In this thesis, 2-dimensional (2-D) gel electrophoresis coupled with Western-immunoblotting, mass spectrometry, and bioinformatics were used to identify proteins on the surface of the bacteria that interact *in vitro* with pig intestinal cells (IPEC-1) and have immunogenic properties. Eleven immunogenic bacterial proteins were detected of which LI0710 (annotated as fliC), LI1153 (annotated as Putative protein N), LI0649 (annotated as autotransporter), and LI0169 (OppA; annotated as ABC dipeptide transport system) were predicted to be expressed on the outer membrane. The genes coding for these four proteins were cloned and expressed in *Escherichia coli* and the corresponding recombinant proteins were purified using affinity chromatography. Porcine hyperimmune serum against whole *L. intracellularis* lysate established that all four recombinant proteins were immunogenic.

To be able to quantify invasion of the vaccine strain of *L. intracellularis* in McCoy and IPEC-1 cells and to determine whether antibodies specific for the recombinant *L. intracellularis* proteins inhibit the bacterial attachment and penetration into eukaryotic cells, the bacteria were labeled with cell-permeable fluorescent dye 5'-carboxyfluoroscein succidyl ester (CFSE) prior to cell infection. Flow cytometry was applied to determine the percentage of eukaryotic cells which were infected with fluorescent bacteria. As obligate intracellular bacteria, their replication is

dependant on eukaryotic cells and thus qPCR analysis was applied to quantify bacterial growth. qPCR analysis showed increase of bacterial DNA over the course of five days, indirectly showing that bacterial invasion and growth took place. The $CFSE^{+}$ (i.e. infected) McCoy cells were sorted from the $CFSE^{-}$ (i.e. non-infected) McCoy cells using fluorescence-activated cell sorting (FACS) while confocal microscopy was performed to visually confirm bacterial invasion and cytosolic localization of CFSE-*L. intracellularis.* Rabbit hyperimmune serum was generated against each recombinant protein and we investigated the effect that hyperimmune sera had on invasion of IPEC-1 cells. Serum antibodies significantly inhibited invasion and replication of CFSE-bacteria, thus indicating that each of the recombinant proteins is a potential neutralizing antibody target and a candidate for subunit vaccine formulation. In conclusion, we used 2-D gel electrophoresis coupled with mass spectrometry to identify four antigens that, when formulated in a vaccine, may lead to production of neutralizing antibodies and disease protection.

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DEDICATION

To my Daughter and Wife

For endless love, support, laughter and life joy that you have given me along this endeavor

And

To my Father, Mother and Sister

Who instilled in me true values and encouraged my thirst for knowledge that shaped my life

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2-DE	Two dimensional gel electrophoresis
Ab	Antibody
ABC transporter	ATP-binding cassette transporter
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADIN	Antibody-dependent intracellular neutralization
ADP	Adenosine diphosphate
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AID	Activation-induced cytidine deaminase enzyme
AKT	Protein Kinase B
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activation protein 1
APC	Antigen presenting cell
APC protein	Adenomatous polyposis coli protein
APRIL	A proliferation-inducing ligand
ATOH1	Atonol homolog 1
ATP	Adenosine triphosphate
BAFF	B-cell activating factor of TNF family
BCA	Bicinchoninic acid
BCR	B cells receptor
B. wadsworthia	*Bilophila wadsworthia*
BMDC	Bone marrow dendritic cells
C. albicans	*Candida albicans*

CBPP	Contagious bovine pleuropneumonia
CCK	Cholecystokinin
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDCs	Conventional DCs
cDNA	Complementary DNA
CDPs	Common DC precursors
CFDA	Carboxyfluorescein diacetate
CFSE	5’-carboxyfluorescein
Citrobacter rodentium	*C. rodentium*
CKI	Casein kinase I
CMI	Cell-mediated immunity
CRP	C reactive protein
CSR	Class switching recombination
CT	Cholera toxin
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T lymphocyte antigen 4
CTR1	Copper uptake protein 1
DAMPs	Damage-associated molecular patterns
DAPI	4’,6-diamidino-2-phenylindole
DCs	Dendritic cells
D. desulfuricans	*Desulfovibrio desulfuricans*
DLNs	Draining lymph nodes
DMEM	Dulbecco's Modified Eagle Medium

DNA	Deoxyribonucleic acid
DOC	Sodium deoxycholate
dpi	Days post-infection
Dr.	Doctor
DSS	Dextran sodium sulfate
E. coli	*Escherichia coli*
EGF	Epidermal growth factor
ESI LC-MS/MS	Electrospray ionization liquid chromatography mass spectrometry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
EPE	Equine proliferative enteropathy
ESI MS/MS	Electrospray mass spectrometry
FACS	Fluorescence-activated cell sorting
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
Flt3 ligand	Fms-related tyrosine kinase 3 ligand
FOXP3	Forkhead box P3
FSC	Forward scatter
GAD	Glutamate decarboxylase
GALT	Gut-associated lymphoid tissue
GC	Germinal centre

G-CSF	Granulocyte-colony-stimulating factor
GIP	Gastric inhibitory polypeptide
GM_1	Monosialotetrahexosylganglioside
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK3	Glycogen Synthase Kinase 3
HBV	Hepatitis B virus
HCD	Higher Energy Collision-induced Dissociation
H&E	Hematoxylin and eosin
H. pylori	*Helicobacter pylori*
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSC	Hematopoietic stem cells
IBD	Inflammatory bowel disease
iDCs	Immature DCs
IECs	Intestinal epithelial cells
IEF	Isoelectric focusing
IF	Immunofluorescence
IFA	Incomplete Freunds Adjuvant
IFAT	Indirect antibody fluorescent test
IFN	Interferon
IFNGR	Interferon-gamma receptor
IgA	Immunoglobulin A
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgM	Immunoglobulin M

IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
IL	Interleukin
ILFs	Isolated lymphoid follicles
i.m.	Intramuscular
iNOS	Inducible nitric oxide synthase
ingLNs	Inguinal lymph nodes
IPG	Immobilized pH gradient
IPMA	Immunoperoxidase monolayer antibody assay
IRDye	Infra-red dye
ISC	Intestinal stem cell
ISG	Interferon stimulated genes
kb	kilobase
kDa	Kilodalton
KGF-1	Keratinocyte growth factor 1
Ki67	Protrein encoded by the *MKI67* gene
L. monocytogenes	*Listeria monocytogenes*
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
L. intracellularis	*Lawsonia intracellularis*
LDC	Langerhans dendritic cell
LGR5+	Leucine-rich repeat-containing G-protein coupled receptor 5
LPS	Lipopolysaccharide
LP	*Lamina propria*
MALT	Mucosal-associated lymphoid tissue
M cells	Microfold cells

MCSF	Macrophage colony-stimulating factor
mDCs	Mature DCs
MDP	Macrophages and DC precursor
MenB	*Neisseria meningitidis* serogroup B
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLN	Mesentery lymph node
Mmm	*Mycoplasma mycoides* subsp. *mycoides*
MNV	Murine norovirus
MOI	Multiplicity of infection
MPs	Myeloid precursors
mRNA	Messenger Ribonucleic acid
MS	Mass spectrometry
MUC	Mucine protein
MyD88	Myeloid differentiation factor 88
MW	Molecular weight
NAIP	NLR family, apoptosis inhibitory protein
nanoLc	Nanoscale capillary liquid chromatography
NFκB	Nuclear factor κB
NICD1	Notch-1 receptor
NK	Natural killer (cells)
NKT	Natural killer T cells
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NRP1	Neuropilin 1

OD	Optical density
OMV	Outer membrane vesicles
ORF	Open reading frame
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PAS	Periodic Acid Schiff staining
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PCV2	Porcine circovirus type 2
pDC	Plasmacytoid dendritic cell
PE	Proliferative enteropathy
PEDV	Porcine epidemic diarrhea virus
PHE	Proliferative hemorrhagic enterpathy
pI	Isoelectric point
p.i.	Post-infection
PIA	Porcine intestinal adenomatosis
pIgR	polymeric immunoglobulin receptor
PP	Peyer's patches
PPE	Proliferative porcine enteropathy
PRR	Pattern recognition receptor
PRRS	Porcine reproductive and respiratory syndrome
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RALDH	Retinal dehydrogenase

RIG	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROC	Receiver operating characteristic
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-qPCR	Quantitative reverse transcriptase PCR
RT-PCR	Real time polymerase chain reaction
RV	Reverse vaccinology
SAA	Serum amyloid A
S. agalactiae	*Streptococcus agalactiae*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide-based discontinuous gel
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
SIgA	Secretory immunoglobulin A complex
SC	Secretory complex
s.c.	Subcutaneous
SCFA	Short acid fat acids
SLAM7	Signaling lymphocytic activation molecule
SoELISA	Sonicated bacteria ELISA
SOX9	Protein encoded by *SOX9* gene
SPF	Specific pathogen free
SPI2	*Salmonella* pathogenicity island 2

S. pneumoniae	*Streptococcus pneumoniae*
SSC	Side scatter
STAT	Signal transducer and activator of transcription
S. Typhimurium	*Salmonella* Typhimurium
T3SS	Type 3 secretion system
TA cells	Transit amplifying cells
T-bet	T-box transcription factor expressed in T cells
TβRII	TGF-β receptor
$TCID_{50}$	Tissue culture infectious dose 50
TCR	T cell receptor
TCF4	T cell factor 4
TGFβ	Transforming growth factor beta
Tfh	T follicular helper cell
Tfr	T follicular regulatory cell
Th1	T-helper lymphocyte type-1
Th2	T-helper lymphocyte type-2
Th17	T-helper lymphocyte type-17
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrotic factor alpha
Treg	T regulatory cell
TUNEL	Terminal deoxyuridine nick-end labeling method
UPEC	Uropathogenic *E. coli*
WB	Western blot
Wnt	Wingles/Integrated family of signaling glycoproteins

WS	Warthin Starry silver staining
WT	Wild type

1. LITERATURE REVIEW

1.1 PROLIFERATIVE ENTEROPATHY

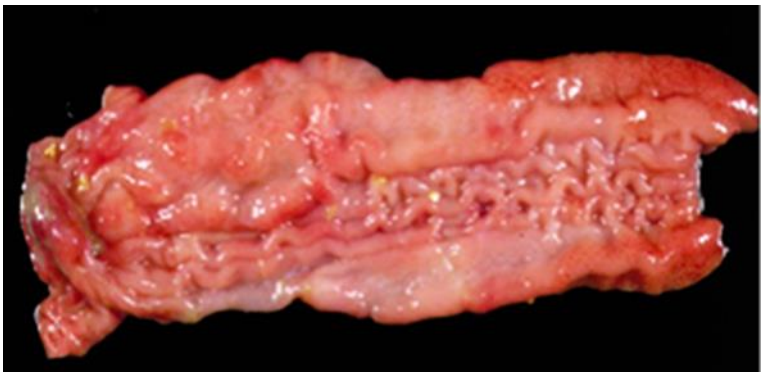
1.1.1 Etiology and clinical findings in different animal species

Proliferative enteropathy (PE) is an enteric infectious disease caused by *Lawsonia intracellularis*, which is an obligate intracellular, Gram-negative bacterium (1). This bacterium is endemic with wide geographical distribution in swine herds all over the world. PE is a major cause of weight loss and mortality in pigs and presents significant economic burden and losses for the swine industry. PE has been diagnosed in other animal species, horses, especially foals (2), rabbits, hamsters, ferrets, dogs, foxes, rats, sheep, deer, emus, guinea pigs, ostriches, and nonhuman primates (3).

The causative agent for PE remained elusive for more than 70 years until 1993 when Lawson's group managed to isolate intracellular bacteria from infected pigs and successfully propagated and maintained them in a rat enterocyte cell line (4). In 1995, this intracellular bacterium was described and a new genus and species was introduced as *L. intracellularis*, in honor of Dr. Lawson (1).

PE in pigs has two distinct clinical and pathologic forms, porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE). Porcine intestinal adenomatosis (PIA) affects young, post-weaned pigs, usually between 6 and 20 weeks of age and the major symptoms of include diarrhea, anorexia and weight loss (5). This form of the disease is mild and it is often subclinical in form with major characteristics including thickening of the wall of intestine due to proliferating immature crypt epithelial cells. Evidence of infection is usually only observed at

slaughter when pathologic lesions can be observed in the alimentary tract, especially in the terminal ileum (Fig. 1.1). Despite these lesions, the pig immune system does not respond to infection with infiltration by inflammatory cells at this stage of the disease, which may indicate that the bacteria have an immunosuppressive effect on the host's immune system (6). Although clinical signs are mild, infected animals shed the bacteria in the feces and are a source of disease transmission. Despite the overt lack of clinical signs of disease, PIA impedes weight gain, thus negatively affects barn profitability, making it an important swine disease.



(Guedes et al 2017, Veterinary pathology Vol. 54(4) 620-628)

Figure 1.1 Proliferative enteropathy, ileum, pig. Corrugation of the mucosa and hyperemia of folds, day 11. Representative image from 5-week-old pigs experimentally infected with 4.37 x 10^9 *L. intracellularis*.

PHE is the acute form of the disease that mainly affects young, adult pigs, 4 to 12 months of age such as finishing pigs, gilts, and boars (5). The main symptoms of PHE are profuse hemorrhagic diarrhea leading to the sudden death of the animals. The pathological findings include thickened and rugose mucosa of the terminal ileum replete with blood in the intestinal lumen (Fig. 1.2). Histological analysis indicates that the ileal crypts have undergone extensive proliferation and there may be evidence of bacteria in the apical cytoplasm of the epithelial cells, in macrophages in the *lamina propria* and submucosa, within epithelial capillaries, and within the

lymphatics (7). An obvious inflammatory response is present in the PHE form of the disease and this inflammatory response may be the reason for more severe symptoms and high mortality rate relative to the PIA form of the disease.

Figure 1.2 Proliferative hemorrhagic enteropathy, ileum from gilt. Corrugation of the mucosa and profuse hemorrhagic inflammation with blood filling the entire length of ileal lumen. Intestine was received from a Saskatchewan pig farm experiencing an outbreak of PE.

Equine proliferative enteropathy (EPE) is intestinal disease in horses, especially foals, caused by *L. intracellularis* strain adapted to horses. These adapted strains of *L. intracellularis* also cause disease in rabbits but do not cause characteristic PE disease in pigs and hamsters (8). The genotypic analysis did not reveal any significant differences between swine and horse isolates and the cause of species adaptability has not yet been elucidated (3).

EPE is characterized by gastrointestinal symptoms that can range from mild watery diarrhea to bloody diarrhea and colic. Except for intestinal symptoms, horses develop lethargy, fever, anorexia, weight loss and peripheral edema, which is a symptom very specific for EPE (3). Edema is localized in lower parts of the body, usually in distal limbs (3). Further, another important

clinical finding in horses is hypoproteinemia due to low serum albumin levels. The normal range of total protein concentration in horses is 5.7-7.9 g/dl and albumin is 2.5-3.8 g/dl but EPE can lead to total serum protein concentrations less than 5 g/dl and 2 g/dl albumin (3). The pathologic mechanism responsible for peripheral edema and hypoproteinemia/hypoalbuminemia is not yet clear but it is suggested that decreased feed intake combined with the deficient absorption of nutrients due to errant enterocyte proliferation and lack of enterocyte differentiation may lead to lower protein uptake and higher protein loss (9).

1.1.2 Infection and pathogenesis

L. intracellularis infection and pathogenesis have been studied in different animal models but understanding this disease has been hampered by the bacteria's obligate intracellular nature, the competition with numerous and diverse commensal bacterial flora, and the complicated and often distinct biochemical environment in different parts of pig's intestine. Its primary route of transmission is oral ingestion of contaminated feces from infected herd mates or rodents. Feces from mice experimentally infected with *L. intracellularis* were able to infect pigs thus showing the potential of rodents to be important factors in transmission of infection to pigs and possibly horses (10). The bacteria have adapted to survive the biochemical and immune defense challenges of a mammalian organism such as the low pH and pepsin in the stomach. The bacteria move with the chyme to the small intestine where the microenvironment is adequate for infection of enterocytes in the middle and distal jejunum and ileum. The possible reasons for preferring jejunum and ileum mucosa for invasion may be the favourable nutrient and pH environment, the presence of specific commensal bacterial flora and/or some yet uncharacterized receptors

expressed on enterocytes in these segments but not in the duodenum or large intestine. The importance of commensal bacteria for *L. intracellularis* infection of pigs have been shown in an experiment where germ-free pigs were resistant to infection with pure *L. intracellularis* culture but were susceptible to infection when commensal flora was restored (11). Commensal bacteria could provide necessary nutrients, pH environment, immune regulation or other stimulatory signals that benefit *L. intracellularis* survival, attachment, and penetration in mucosal enterocytes. Due to the complexity of commensal flora and region-specific distribution patterns, the determinants of infection remain unsolved.

L. intracellularis are obligate intracellular bacteria that must attach to and penetrate cells to be able to colonize and multiply (Fig. 1.3). These bacteria have an affinity towards immature and undifferentiated enterocytes inside jejunal and ileal crypts (Fig. 1.3). Upon infection, *L. intracellularis* arrests epithelial cell differentiation and maturation thus keeping them in a state of continuous proliferation, which benefits bacterial division and spreading. These proliferating cells change the normal architecture of intestinal mucosa, leading to the absence of goblet cells, the destruction of brush border area, and the presence of many proliferating, undifferentiated enterocytes (Described in more detail in **1.3.4 Immune response to infection with *L. intracellularis*)**. Together, these microscopic changes progress to macroscopic evidence of thickened, corrugated mucosal folds often covered with fibrinonecrotic membranes or blood in cases of PHE.

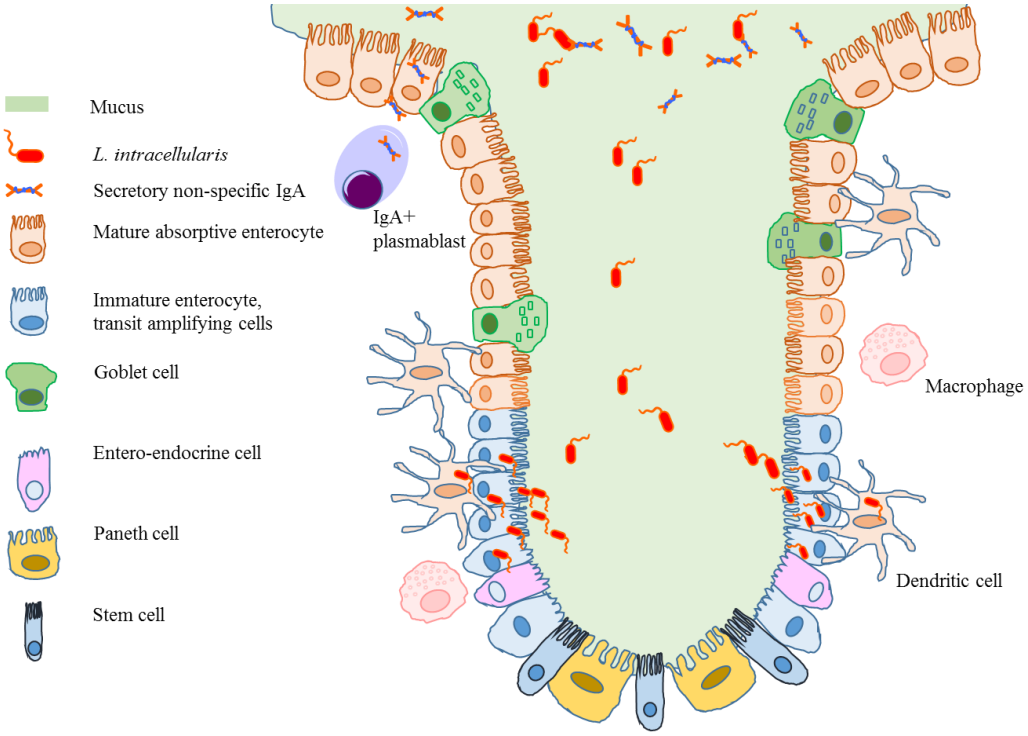


Figure 1.3. Small intestine crypt epithelial cells and initial attachment of *L. intracellularis* to immature enterocytes. *L. intracellularis* are able to survive bactericidal molecules released by crypt intestinal cells and it can persist despite commensal competition. Their motility is controlled by flagella and, using an unknown mechanism, they can traverse through thick mucus and attach to immature epithelial cells inside the crypt. *L. intracellularis* have affinity towards immature epithelial cells, most likely transit amplifying cells (light blue), where they invade and multiply. They impact the eukaryotic cell cycle to inhibit differentiation while inducing proliferation of these immature cells.

There are several studies describing the progression of infection and PE lesions in hamsters but most clinically relevant studies were performed in pig models (12, 13, 7). Boutup and al. 2010 described the early pathogenesis of *L. intracellularis* infection and explored small intestinal loops as a model to study early infection. This group reported that *L. intracellularis* contacts the microvilli of mature enterocytes between 3 and 6 h after surgical inoculation into intestinal loops of pigs (12). In their small intestine loop model, they reported bacterial contact with jejunum enterocytes 6 h after bacterial inoculum into the loop (12). Although interesting, care must be taken to avoid over-interpretation of these results due to the intestinal loop model being an imperfect

model. First, the route of infection is not natural as the bacteria are injected directly into the intestinal loop and this method of infection could impair normal intestinal peristalsis as it concentrates the bacteria in a small area. Second, the creation of ligated intestinal loops could impact local microvasculature and nerves thus potentially changing pH and oxygen concentration inside the loop, which may in turn impact *L. intracellularis* penetration and growth. Despite these limitations, this study shows the usefulness of this intestinal loop model and shows that it has the potential to be used to explore the properties of bacteria during early pathogenesis.

Guedes and al. 2017 (7) has presented the most comprehensive study to date of progression of gross and histologic lesions during *L. intracellularis* infection of 5 weeks old pigs from 1 to 35 days post-infection (dpi) with oral inoculation of a dose of 4.37 x 10^9 *L. intracellularis*. Three animals, two from the challenged group and one from the control group, were euthanized on days 1, 3, 5, 8, 11, 15, 19, 24, 29 and 35 dpi (7). Macroscopic and histologic lesions of PE were first identified 11 days after oral inoculation but the first detection of *L. intracellularis* antigen using immunohistochemistry (IHC) was at 5 dpi (7). These data indicate that bacteria establish themselves in the small intestine during the first 5 dpi and that they need at least 11 dpi to induce characteristic lesions in the small intestine mucosa. The lesions in small intestine on day 15 were characterized as a thickening and corrugation of the ileum and middle jejunum serosa (7). The mucosa of ileum and distal jejunum (1 m length) and colon (5 cm in length) were also affected with characteristic corrugations and thick folds covered with fibrin (7). The most severe lesions were observed in the middle jejunum with areas of mucosal necrosis and findings of fibronectin membranes. Between 9 and 28 dpi all infected animals had watery diarrhea with traces of blood, abdominal lymph nodes were 3 times the normal size and 200 ml of a yellowish transudate was present (7). The peak of gross mucosal lesions and the highest number of bacteria in enterocytes

were recorded between 15 and 24 dpi (7). At necropsy on day 19 after infection, one pig showed progression of macroscopic lesions and spreading further into jejunum and colon. In correlation with the microscopic lesions and number of bacteria present in enterocytes, an apoptotic index was measured using the terminal deoxyuridine nick-end labeling (TUNEL) method and caspase-3 was measured using IHC (7). From 11 to 24 dpi, the apoptotic index increased and then returned to control values at 29 dpi (7). These data correspond well with previous findings by MacIntyre at al. where the peak of infection and the spread of characteristic lesions were detected at the same period in 7 weeks old pigs infected orally with a dose of 2 x 10^8 of *L. intracellularis* (13). This group also explored the immune response 41 dpi and found evidence of immune down-regulation. Guedes et al 2017 reported the detection of the highest titres of antigen-specific IgA in intestinal lavages between 15 and 29 dpi but they did not explore the correlation of protection and the importance of the local humoral response (7). *L. intracellularis* antigens in the intestine could be detected up to 29 dpi but macroscopic and microscopic lesions were resolved (7). Also, after 35 days, no bacterial DNA on intestinal samples could be detected by PCR analysis which indicates complete clearance of the pathogen 35 dpi (7). These studies provide insight into stages of *L. intracellularis* infection and pathogenesis, which added to knowledge of the *L. intracellularis* life cycle.

1.1.3 Diagnosis

Porcine PE was first reported under the name PIA in 1931 by Biester and Schwarte (14). The first diagnosis was based on symptoms, postmortem gross pathology, and microscopic examination of small and large intestine. This diagnosis can be problematic because the

interpretation is subjective and other enteric diseases in pigs may have similar symptoms, macroscopic and microscopic lesions that could interfere with the precise diagnosis of PE. Thus, the true nature and the causative agent of PE was elusive and not known until the invention of more sensitive and specific diagnostic techniques, such as specific *L. intracellularis* DNA detection by PCR and IHC of tissue samples (5, 15).

The *post-mortem* techniques previously used to diagnose PE included using hematoxylin and eosin (H&E) and Warthin Starry (WS) silver staining slices of tissue with characteristic macroscopic lesions. The H&E staining is able to detect the characteristic proliferative changes of immature enterocytes and to confirm the absence of goblet cells. This stain can confirm cell abnormalities in small intestinal tissue but it does not identify the causative agent and it lacks specificity. The WS silver staining can detect curved, rod-shaped intracellular bacteria in intestinal histological samples, but it is also not specific because it can stain other bacterial species. Despite not being specific for *L. intracellularis*, the WS silver stain helped to show that PE is caused by rod-shaped intracellular bacteria and to distinguish PE from viral intestinal diseases. One disadvantage of the WS silver staining is that it cannot detect bacteria in necrotic tissue, which can be one of the forms of PE disease (16, 17, 18).

IHC utilizes specific monoclonal antibodies against *L. intracellularis* to detect bacteria in tissue sections from infected animals. Guedes et al. 2002, compared sensitivity and specificity of IHC, H&E and WS staining of ileum tissue of pigs experimentally infected with *L. intracellularis* (19). IHC showed higher sensitivity of 86.8% compared to 36.8% sensitivity using H&E staining and 50% sensitivity using WS staining (19). The authors concluded that the higher sensitivity of IHC could be explained by the specific binding of the monoclonal antibody to the 21 kDa outer membrane protein of *L. intracellularis* (19|). In contrast to the WS method of staining, IHC can be

used to identify *L. intracellularis* antigens in necrotic intestinal tissues or in cases of resolving stage of infection where the bacteria are found only in the cytoplasm of *lamina propria* mononuclear cells (19).

Histopathology and IHC methods are important diagnostic tools to detect *L. intracellularis* in tissue samples of infected animals but they are not suitable for the monitoring of active infection of pig herds. The serological methods that detect specific antibodies against *L. intracellularis* in animal serum are suitable to detect prior exposure, to survey *L. intracellularis* infected animals within herds, and/or to detect vaccinated animals. The serology assays that are used for this purpose are indirect fluorescent antibody test (IFAT), immunoperoxidase monolayer antibody assay (IPMA) and enzyme-linked immunosorbent assay (ELISA) (5).

Developed by Dr. Lawson and his group (20), IFAT was the first assay to detect *L. intracellularis*-specific IgM and IgA in serum of naturally infected pigs. Later, an IFAT that detected IgG antibodies was developed using bacteria grown in pure culture stained with pig serum as the primary antibody and fluorescin isothiocyanate (FITC) labeled anti-swine IgG was used as the secondary antibody (21). IFAT was used to detect *L. intracellularis* antibodies 21 to 28 days after a challenge with a pure culture, which showed 90% sensitivity and 96% specificity. In addition, IFAT was more sensitive than PCR which only showed 47% sensitivity but less specific than PCR which detected 100% specificity (21).

Immune peroxidase monolayer assay (IPMA) is assay developed as an alternative to IFAT and has similar workflow and procedures with the exception that IPMA anti-swine IgG secondary antibody are labeled with peroxidase and require elimination of endogenous H_2O_2 activity in tissue (22). IPMA uses light microscopy to detect antibodies, which can have an advantage over IFAT

that requires fluorescent microscopy. However, plate preparation is complicated and data interpretation can be subjective leading to discrepancies in results across labs (23). When compared to each other, both assays share a high level of agreement (98%) in controlled infection studies which indicate equal diagnostic usefulness in the detection of *L. intracellularis* seropositive pigs (24).

Numerous enzyme-linked immunosorbent assays (ELISA) have been developed for *L. intracellularis* including indirect ELISA which uses whole cell antigen from sonicated bacteria (SoELISA) (25), sodium deoxycholate (DOC) extracted antigen (26), bacterial targets such as LPS (27), and LsaA protein ELISAs (28). Not all these assays are commercially available nor are they readily available for clinical use in the field. To bridge this gap and to provide a clinically useful assay to detect seropositive animals, commercially-available blocked ELISAs were developed (bioScreen Ileitis Antibody ELISA, Synbiotics Corporation, Lyon, France and SVANOVIR® *L. intracellularis*/Ileitis-antibody, Svanova, Sweden). Blocked ELISA detects anti-*L. intracellularis* antibodies in pig's serum. Antigen are fixed to the bottom of the plate. If serum is positive, anti-*Lawsonia* antibodies will bind to the fixed antigen and the remaining serum components will be rinsed away (i.e. blocked). If there are no specific antibodies present in serum, antigen will remain free to bind to horseradish peroxidase (HRP)-conjugated monoclonal anti-*Lawsonia* antibody which will result in a distinctive blue colour. Pigs that have been exposed to *L. intracellularis* or have been vaccinated against *L. intracellularis* will have sera with specific anti-*Lawsonia* antibodies present and results will show a weaker reaction and a lighter colour. The reaction optical density (OD) is measured in an ELISA plate reader and the percentage of inhibition is calculated. An OD above 30 is considered positive, between 20 and 30 is questionable and below 20 is considered negative. Blocked ELISAs allow the direct quantification of data, the ability to test 184

samples in one plate, the removal of subjective bias across labs and they are major advantage over other serological tests to detect animals with *L. intracellularis* antibodies in their sera.

The commercial blocking *L. intracellularis* ELISA has been compared to the IFAT, IPMA and LPS ELISA to assess agreement, sensitivity, and specificity to this bacterium (29, (30) (23). Compared to IFAT, the sensitivity of the blocking ELISA was 72% and the specificity was 93% (calculated with Bayesian modeling techniques to account for the absence of true gold standard) (29). In another study using different statistical models, the reported sensitivity of the blocking ELISA was 90% and with a 83% specificity with significant association between tests as determined using Fisher's exact Chi-square test (P = 0.0001), kappa test 0.74 (P< 0.0001, one-tailed test) and McNemar's Chi-square test (3.84) (30). The results from this study suggest that there is a high agreement between the two assays and that users of the commercial blocking ELISA can be confident in its sensitivity and specificity. In the study by Magtoto et al 2014 (23), the blocking ELISA sensitivity was compared to the IPMA and LPS-ELISA. The three assays showed a 95 % correlation. Compared to IPMA, the commercial blocking ELISA showed 91% sensitivity and 100% specificity whereas the non-commercial LPS-ELISA was 95% sensitive and 100% specific (23). The IPMA assays showed the highest sensitivity and specificity of all tested serological assays but the blocked ELISA is the most practical for herd surveillance because it is a high throughput assay, easy to use, economical, and has high sensitivity and specificity.

Serologic tests are useful to monitor exposure to *L. intracellularis* and seroconversion of animals, but they cannot distinguish between animals vaccinated with live-attenuated or killed vaccine and those naturally exposed to the bacteria. Serum antibodies against *L. intracellularis* do not necessarily correlate with lesions or clinical manifestation caused by these bacteria. To be able

to demonstrate that clinical symptoms of ileitis are caused by *L. intracellularis*, the molecular technique PCR must be used.

PCR can successfully detect *L. intracellularis* in feces or in intestinal tissue of infected pigs. It is an antemortem assay that is highly sensitive, highly specific and a relatively economical method to detect even low levels of intracellular microorganism (31) (32). Several PCR methods have been developed for *L. intracellularis* detection: conventional, nested, PCR-ELISA, real-time PCR, multiplex and quantitative RT-PCR. Diagnostic performance of multiple fecal *L. intracellularis*-specific PCR assays for PE in pigs across seven publications was evaluated by Pedersen et al 2010 (32). Four studies assessed PCR analysis under experimental conditions while three studies evaluated diagnostic sensitivity/specificity of PCR under field conditions (32). This study presents data of different PCR tests including conventional, nested, real-time and multiplex-based OCR with the reported range in diagnostic sensitivity (36–100%) and specificity (50–100%) (32). The nested PCR techniques are considered to be the most sensitive but in this study they showed equal sensitivity to conventional PCR (32). The studies had technical differences that could interfere with objective comparison of sensitivity/specificity such as variation in sample sizes, DNA extraction methods and PCR conditions (32). Despite these discrepancies the authors concluded that under conditions where up to 30% of the pigs in a herd have histologic lesions from PE, the risk of false positives will usually exceed the risk of false negatives when applying the PCR tests (32). PCR could detects shedding of *L. intracellularis* from 7 to 8 dpi after infection and that shedding could was observed for 15 days or, in some reported subclinical cases, up to 12 weeks (32). In experimentally infected pigs, PCR could detect shedding of *L. intracellularis* as early as 3 dpi (7). Taken together, these data confirm high sensitivity/specificity of PCR to detect

L. intracellularis in feces of infected animals early in course of infection and also, to monitor prolonged shedding even in subclinical cases.

The presence of *L. intracellularis* in fecal material was established using a quantitative PCR (qPCR) assay using SYBER green (33) with primers specific for the target gene, aspartate ammonia-lyase (*asp*A). This qPCR assay could detect bacterial DNA from spiked fecal samples in concentration of between 2.55 x 10^4 and 2.55 x 10^3 *L. intracellularis*/g (33). Under field conditions, *L. intracellularis* was detected by qPCR in feces from 91 pigs (29.1%) (32) where an nonparametric receiver operating characteristic (ROC) analysis provided an area under the ROC curve (0.93) and an optimal cutoff value of 4.8 $\log_{10}$ *L. intracellularis* bacteria/g feces. This cut-off provided a diagnostic sensitivity of 0.84 and diagnostic specificity of 0.93 (32). This study concluded that performance of fecal qPCR testing for detection of pigs with *L. intracellularis*–associated PE under field conditions was adequate and that fecal qPCR analysis can be used as an accurate diagnostic tool.

In summary, diagnosis of PE and detection of the causative agent *L. intracellularis* can be established using a combination of serology, histology, and molecular techniques. Serum antibody titres indicate previous exposure to *L. intracellularis* but not necessarily current infection. Histology can be used to identify characteristic microscopic lesions and IHC can be used to identify *L. intracellularis* in enterocytes. However, because these histological techniques must be performed *post-mortem*, they cannot be used for surveillance of PE in herds. Instead, PCR analysis of feces is useful to monitor health status of the animals but it can detect bacteria only when animals are shedding which may mean that some non-shedding but subclinical infected animals may be missed. Detection of subclinical infected animals is still a challenge for available diagnostic techniques and further research is needed to find adequate diagnostic solutions. Finally,

there is no current diagnostic test that can distinguish between vaccinated from infected animals, which poses problem in the inaccurate assessment of PE prevalence and herd infection status. Currently, PCR of feces or tissue and IHC of intestine are considered the standard tests for PE diagnosis (15). The serological results and molecular tests are necessary to monitor prevalence and disease spread and to implement proper management measures to treat or prevent occurrence of PE.

1.1.4 Epidemiology

L. intracellularis has been found in swine herds all around the world. Serological data indicate that US (96%) and Northern Europe (70-90%) have a higher prevalence of *L. intracellularis* than Southern Europe (50-70%) (34). Evaluation of *L. intracellularis* antibodies using specific blocking ELISA in intensive pig farms in China revealed that the true prevalence (i.e. prevalence corrected for the imperfect sensitivity and specificity of the testing method) was 77% (35) and the highest prevalence was detected in fattening pigs, breeding sows and boars and in Northern or Southern regions where intensive pig production is highest (35). These data correspond to a serological investigation in Australia and Korea, where high prevalence was detected among intensive production systems. In Australia, commercial blocking ELISA indicated 72% seroprevalence for Western Australia and 88% for Queensland, while the mean percentage of seropositive animals within the herds was 84% (36). A study from South Korea reported seroprevalence in growing and fattening pigs of 45% and 59%, respectively, while 100% of herds had seropositive animals (37). These results should be taken with caution because the Korean study

used IFAT assay to detect *L. intracellularis*-specific antibodies, which has not been standardized and may lead to sizable variations across different laboratories.

When PCR was used to detect bacteria in five large pig intensive production farms in Denmark, all examined herds were seropositive for *L. intracellularis* and 75% of animals were infected (38). The results from PCR testing of nursery herds in Sweden revealed that 48% of herds were actively infected with *L. intracellularis* (39). These data confirm worldwide distribution of causative agent of PE. PCR can be used to detect *L. intracellularis* in feces, which can positively identify individual animals that actively shed bacteria and are a source of infection. Studies in the field and in controlled challenge exposure showed that the PCR method detected bacteria in pig feces 1-2 weeks before serum antibodies could be detected with IPMA (40, 19). These data indicate that adequate timing and application of diagnostic tests are very important for acquiring representative data. Positive animals identified by serology or PCR may not show the clinical signs of PE or may only be determined as positive by one diagnostic method. In a recent study where authors followed the progression of lesions associated with PE in experimentally infected pigs, they detected fecal shedding using PCR as early as 3 dpi and detection in feces remained positive up to 35 dpi (7). IgA secretion in intestinal lavage was detected at day 15 and was still detectable up to day 29 (7). Although this study did not evaluate serum antibodies, it indicates that PCR analysis may be a sufficient diagnostic method to detect early stages of infection prior to induction of humoral immunity. In the case where pigs tested positive via serological methods but negative with fecal PCR, animals did not actively shed bacteria but were previously exposed to bacteria (41). Data from different studies where seroprevalence and bacterial presence in feces were examined emphasize the need of applying both serology and PCR to generate representative data that could be used in the prevention and better management of PE.

The high prevalence and global distribution of *L. intracellularis* reflect the superb capability of these bacteria to be transmitted among animals. Transmission may occur through three main sources: 1) via fecal material that persists due to poor sanitation measures and by housing of large numbers of susceptible animals of different ages in small spaces (42, 43, 2) by persistence of bacteria in sows that act as subclinical carriers (44); and 3) transmission by rodents present in and around pig farms (45, 10). The fecal-oral route is the mode of transmission that provides bacteria an efficient mode to spread among susceptible animals as the bacteria can survive outside the host and remain infectious for two weeks in feces at 5-15 ^{o}C (46). The infectious dose in feces is $10^4 – 10^6$ *L. intracellularis* but because infected pigs can shed 7 x 10^8 bacteria per g of feces, the fecal material is highly likely to be adequate for transmission (47). Subclinically infected animals can shed bacteria intermittently for extended periods of time (at least for 10-12 weeks after initial infection in asymptomatic experimentally infected pigs) (48, 49). The ease by which the bacteria can be transmitted via feces stresses the importance of proper hygiene, sanitary and disinfectant measures in pig farms. Slatted concrete flooring is a major factor aiding in the high prevalence of *L. intracellularis* seropositive pigs (42) and animals raised in modern intensive production systems tend to have higher seropositive prevalence than animals raised outdoor (42, 37, 36, 35). The modern pig production systems tend to intensify production by adding more animals in limited spaces thus providing ideal conditions for *L. intracellularis* to spread through animal to animal contact or via feces and that allowing adequate cleaning, disinfecting and drying time is necessary to promote proper sanitation (46). Biosecurity is important in preventing the introduction of *L. intracellularis* into farms and quarantine showed efficacy in keeping some herds free from PE (50, 39). The role of rodents in *L. intracellularis* transmission had been speculated due to bacteria capability to infect hamsters (51), mice and rats (52, 53, 54), and the prevalence of

rodents in and around pig farms (45). A study showed as high as ≥70.6% of wild rats captured in pig farms were infected with *L. intracellularis* and some specimen shed a high number of bacteria per gram feces (10^{10} per g) (45). The duration of fecal shedding in experimentally infected mice and rats from the same study persisted for 14-21 days and humoral immunity in both species lasted for 40 days (45) suggesting that these animals could readily infect pigs through their contaminated feces. Mice fed *L. intracellularis*–infected pig feces became infected and actively shed bacteria in feces (10^4 bacterial cells per day) (10). In turn, pigs that consumed the rodent feces were infected and shed an average 10^4 bacteria per g of feces (10). These studies indicate that rodents may act as reservoirs for bacteria and they may play an important role in transmission of *L. intracellularis.*

Persistence of *L. intracellularis* in sows with subclinical disease are a major source of bacterial transmission within the herd (44). Sow group housing increases the transmission of bacteria between sows and piglets and this housing practice can increase the number of shed bacteria in feces within rooms (50). The age and parity of the sows are important factors that impact bacteria shedding (42). Older sows with 3-5 parity or more are associated with fewer numbers of seropositive offspring whereas offspring from gilts have increased risk of being seropositive after 5 to 26 weeks (55). These results suggest that higher parity sows have developed protective immunity to *L. intracellularis* which can benefit the offspring through passive immunity or that these multiparous sows may shed lower numbers of bacteria that are insufficient to cause infection. The role of passive immunity in protection of piglets is not clear although sows with anti-*L. intracellularis* serum antibodies that do not shed the bacteria have been shown to protect offspring suggesting that passive immunity may be protective (22, 44).

Transmission between herds occurs mainly through the introduction of infected animals into a naïve herd. Newly introduced subclinically infected replacement stock experience stress

during transport and by being introduced to a new environment. This increased stress may result in them actively shedding *L. intracellularis* into the barn, which may lead to infection of co-housed animals.

1.1.5 Treatment and management of PE

The global prevalence of PE in wildlife, in backyard, farmed pigs, and in intensive pig systems poses a challenge in the control, treatment, and eradication of this disease. Although PE is not as devastating to pig herds as other pig diseases such as porcine reproductive and respiratory syndrome (PRRS), porcine epidemic diarrhea virus (PEDV) or Classical or African Swine Fever, the economic losses due to subclinical infections and/or outbreaks after stress remain a great challenge for pig farmers. Biosecurity measures coupled with treatment and vaccination are the best currently available solutions to control *L. intracellularis* transmission. Modern pig farming has adopted management practices that can lower the possibility of bacteria transmission such as increasing sanitation and disinfection, applying an 'all in, all-out' system for herds, and using older sows with higher parity in rotation (42).

Rodent control and stringent biosecurity including thorough cleaning and disinfection of animal spaces are important management practices to prevent transmission of *L. intracellularis* in pig farms (45, 10, 42). The most effective disinfectants include quaternary ammonium compounds and povidone-iodine (46). For cleaning and disinfection to be successful, it is important to leave sufficient time for the disinfectant to work before populating pens with animals (46). Thus, the practice of all in-all out husbandry in pig farms has shown to be associated with lower risk of PE in herds (42). Segregated early weaning, medicated early weaning, and the establishment of high

health herds free of *L. intracellularis* are other management measures that reduce the risk of PE in herds (5). Finally, the association of farm building type, type of production system and materials used in construction can contribute to increased risk of PE (56, 42). For example, slatted concrete flooring was associated with a higher risk of infection due to inefficient cleaning and disinfection of those types of floors (42) with the alternative of straw bedding and solid floors leading to reduced disease prevalence.

Animals with PE can be treated with administration of antibiotics into animal feed or via intramuscular injections. Ideally, antibiotics should have high efficacy and specificity against *L. intracellularis*, adequate pharmacodynamics, they should be distributed throughout the entire gastrointestinal tract with intracellular absorption and retained activity, all the while being cost-effective (15). Evaluation of antimicrobial activity of different antibiotics on *L. intracellularis* has been a challenging task due to the obligate intracellular nature of these bacteria and the difficulties in isolating and maintaining them, *in vitro*. A study from 2009 tested the antimicrobial activity of select antibiotics on ten *L. intracellularis* isolates from North America and Europe (57). They reported that carbadox, tiamulin, and valnemulin displayed the highest intracellular bactericidal activity (0.5 mg/ml minimum inhibitory concentrations (MICs)) against six *L. intracellularis* isolates from North America (57). Chlortetracycline and tylosin had moderate bactericidal activity against *L. intracellularis* (0.125 to 64 mg/ml and 0.25 to 32 mg/ml MICs, respectively) (57). Lincomycin had the lowest bactericidal activity against the majority of *L. intracellularis* isolates with a MIC range from 16 to >128 mg/ml (57). Valnemulin had highest bactericidal activity against extracellular *L. intracellularis* with MICs ranging from 0.125 to 4 mg/ml followed by carbadox (4 to 32 mg/ml MICs), chlortetracycline (32 to 64 mg/ml MIC), tiamulin (1 to 32 mg/ml MICs), tylosin (1 to >128 mg/ml MICs) and lincomycin (>128 mg/ml MICs) (57). Antibiotics with

no antimicrobial activity against *L. intracellularis* included aminoglycosides and aminocyclitols including neomycin and gentamicin (58). Because neomycin and gentamycin suppress the growth of other enteric bacteria without interfering with *L. intracellularis* survival, they are used in medium for *L. intracellularis* isolation and *in vitro* growth (4).

Another study using four European isolates of *L. intracellularis* showed similar susceptibility to carbadox, tiamulin, and valnemulin as the North American isolates including tylosin with a MIC range of 0.5–2 mg/ml, chlortetracycline with a MIC range of 0.25–16 mg/ml and lincomycin with a MIC range of 8–64 mg/ml (57). Valnemulin had the highest extracellular activity against *L. intracellularis*; all isolates had MICs of 0.25 mg/ml (57). Antimicrobials that had moderate activity against *L. intracellularis* included tiamulin with an MIC range of 1–4 mg/ml, carbadox with an MIC range of 1–4 mg/ml, tylosin with an MIC range of 2–16 mg/ml and chlortetracycline with an MIC range of 16–64 mg/ml (57). The antimicrobial that showed the lowest activity against *L. intracellularis* was lincomycin with MICs of 32–128 mg/ml (57). Based on these results, the authors suggest that carbadox, tiamulin, and valnemulin are the most active antimicrobials, followed by chlortetracycline and tylosin which have moderate antimicrobial effect with lincomycin having the least antimicrobial effect against *L. intracellularis* (57). Because the antimicrobial activity was performed on bacteria grown in cell culture, the antimicrobial activity of the antibiotics may or may not show comparable activity under field settings in animals with a clinical case of PE.

Pig farms with cases of PE commonly treat infections with tiamulin at 120 ppm, tylosin at 100 ppm, lincomicyn at 110 ppm, or chlortetracycline (CTC) at 300 ppm given orally mixed with feed (59). Elimination of *L. intracellularis* (confirmed by PCR and ELISA analysis) was achieved in 11 Danish farms with nine farms showing no clinical symptoms for PE. Specific pathogen-free

(SPF) gilts were treated with tylosin (5 mg per kg body weight) for 14 days followed by washing them with a 2% Vicron solution (60). However, even with strict biosecurity measures in place, most farms became re-infected within 1.5 years with only 2 farms stayed free of *L. intracellularis* for 5 years (60). Despite reinfection, animals from re-infected farms had improved feed conversion rates and increased average weight gain in excess of 100 grams over animals from the farms where animals were fed with medicated feed to control ileitis (60) indicating that reinfection did not have a significant effect on animals in these herds.

Currently, there are two commercially available vaccines on the market. Enterisol® is a live avirulent vaccine from Boehringer Ingelheim which has been available since 2004. Porcilis is an inactivated whole cell vaccine available since 2016 from Merck Animal Health. They are an important part in all prevention measures on farms all around the world and present an efficient and economical solution for limiting the effect of PE on pig herds. Their properties and protection characteristics are discussed in Section 1.3.5: Immune protection against *L. intracellularis* infection.

Despite all available prevention measures and treatment options discussed here, *L. intracellularis* is still prevalent in intensive pig productions around the world. The omnipresence of this bacteria and increased pressure to reduce the use of antibiotics in food-producing animals have influenced all involved parties in the pig industry to actively seek and implement new methods to control PE. Biosecurity measures, disinfection, rodent control, modification of diets and use of vaccines have shown promising results in the prevention of PE and eliminating the use of antibiotics.

1.1.6 Economic impact and importance of PE for the swine industry

PE has emerged as an important disease in pig farms with significant associated economic losses. Accurate estimation of economic losses has been challenging to accomplish due to the complexity of PE disease and because most of the studies simply cite approximations of the economic impact. Cost increases contributing to PE are likely due to higher feed consumption, longer facility time to reach market weight, loss of animals, antibiotic treatment costs and reduced reproduction rates in infected sows/gilts (55).

PE has an economic impact on breeding pigs, weaners and finishing pigs. Losses derived from poor growth in fattening pigs suffering from PIA and the associated elevated mortality and treatment costs have been estimated in the UK as $3-11 USD per growing pig and the $3-6.5 million USD total annual cost (61, 62, 63). The annual cost to pig producers in the US has been estimated at 20 million USD (63). McOrist et al 2005 and Mauch and Bilekei 2005 estimated that financial losses in the UK exceed €100 (121 USD) per affected breeding pig which extrapolates to an additional €0.50 (0.61 USD) per growing pig (64, 55).

In Denmark, the banned use of antibiotics in the feed as growth promoters for production animals has had a significant impact in management practices in pig industries. With reduced antibiotics in feed, there was an increased prevalence of diarrhea due to *L. intercellularis* which in turn led to decreased production performance of 18 g to 50 g per day in daily gain and reduced mortality by an extra 0.6% to 1.5%, especially in the weaner period (44). The use of therapeutic antibiotics to treat the infection was estimated to cost between, 1 to 3 USD per animal (44) which amounted to less than 50% of the amount of antibiotics used before the ban (44). These data indicate the importance of applying adequate management practices such as stringent biosecurity

measures and finding alternative to antibiotics to protect against disease. Vaccines offer the most cost-effective preventive measure, which can protect animals with the least economic burden to pig producers while minimizing the use of antibiotics as therapeutics.

1.2 *LAWSONIA INTRACELLULARIS*

1.2.1 Taxonomy

Identification and characterization of *L. intracellularis* was hindered by its intracellular niche where it thrives as well as the inherent difficulties in isolating and growing this bacterium *in vitro*. For years, PE was thought to be caused by *Campylobacter spp*., which were often isolated and cultured from PE cases but it failed to induce characteristic symptoms of PE when infecting conventional pigs (65). DNA restriction enzymes and probe analysis by McOrist 1990 and Gebhart 1991 showed that the organism responsible was not *Campylobacter spp*. so it was described as '*Campylobacter*-like' or 'ileal symbiont intracellularis' (66, 67). This nomenclature was used until 1995 when McOrist at al. sequenced the 16S DNA and finally classified and named bacteria as *Lawsonia intracellularis*, in honor of Dr. Lawson (1).

L. intracellularis belongs to delta subdivision of Proteobacteria in the Desulfovibrionaceae family (68). Genetically, the closest relative of *L. intracellularis* is *Bilophila wadsworthia* showing 92% similarity of 16S ribosomal sequence (3). Interestingly, *B. wadsworthia* is an extracellular anaerobic human pathogen that constitutes less than 0.01% of the normal human gut microbiota but is the third most common anaerobe isolated from intestinal samples from patients with perforated and gangrenous appendicitis (69). It is asaccharolytic and characterized by its strong

production of hydrogen sulfide, and growth stimulation by bile (oxgall) and pyruvate (69). More than 85% of strains demonstrate β-lactamase production when pyruvate-containing media was used for bacterial growth (69). Although *B. wadsworthia* shares 92% similarity with 16S ribosomal gene from *L. intracellularis*, their cultivation, growth, and pathogenesis are markedly different.

L. intracellularis is 91% genetically similar to *Desulfovibrio desulfuricans*, a sulfate reducing bacteria usually found in water, soil and in feces (3, 70). It encodes mechanisms of mercury methylation which could be of importance to protect itself from the environment (70). *L. intracellularis* does not have the capability of reducing sulfate nor methylate mercury thus differing from *D. desulfuricans*. Phenotypic differences also exist between these two species. *L. intracellularis* are acid-fast when stained with modified Zhiel-Nielsen and the *L. intracellularis* pellet is a pale tan colour. *D. desulfuricans* are not stained with modified Zhiel-Nielsen and its pellet is black in colour. Despite using 80 different media that were known to support *D. desulfuricans* growth, *L. intracellularis* could not be cultivated (1). This research shows that *L. intracellularis* is unique obligate intracellular bacteria with unique specific characteristics and pathogenesis.

1.2.2 Characteristics of *L. intracellularis*

L. intracellularis are curved, non-spore forming, microaerophilic obligate intracellular organisms that replicates by binary fission and cannot grow in cell-free media (31). It is a rod-shaped bacterium 1.25-1.75 μm in length and 0.25-0.43 μm in width (1). Trilaminar outer membrane and unipolar flagella were observed in extracellular *L. intracellularis* that helps in propulsion and translocation during infection (6). *L. intracellularis* have one chromosome

(1,475,619 bp) and three plasmids (plasmid A, 27,048 bp; plasmid B, 39,794 bp; and plasmid C 194,553 bp) (71).

Seventeen isolates *L. intracellularis* have been reported so far (3) but they are considered to belong to one species with no major phenotypic or genetic differences yet discovered. There are some specificities of isolates derived from pigs and horses and proof of species adaptability of these strains. For example, foals and pigs infected with species-adapted isolates showed characteristic clinical symptoms, longer times of bacterial shedding and stronger immunological response (72). In contrast, foals challenged with porcine isolate or pigs challenged with an equine isolate showed milder or no clinical symptoms, low levels of bacterial shedding and lowered immunological response to infection (72). This species adaptability expands to rabbits and hamsters, which are susceptible to specific isolates and manifest the clinical symptoms of equine or porcine PE, respectively. Rabbits are susceptible to equine isolates and show characteristic symptoms thus are a good model for equine PE (73). A comparative genome sequencing study on porcine and equine isolates reported an 18-kb prophage-associated genomic island in the pathogenic variant of the porcine isolate which was missing from the equine isolate and from the cell-culture-adapted non-pathogenic-variant porcine isolate (73). This genomic island was absent from isolates obtained from 21 clinically affected horses and four wild rabbits trapped from this horse farm (73). This research was the first reporting genetic difference between two isolates. However, data suggest that this genomic island is not essential for pathogenesis but is speculated to have potential benefits for adaptation of porcine isolate to the host (73).

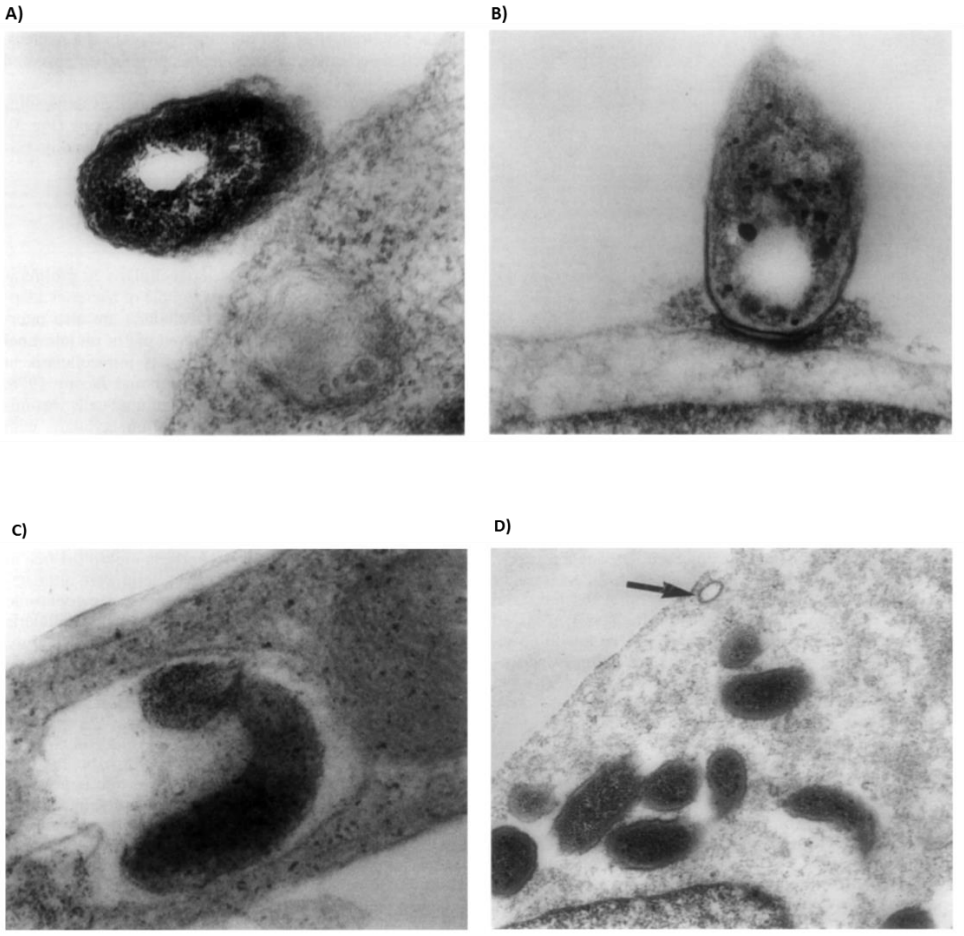
Attenuation of virulence of *L. intracellularis* occurs between 20 and 40 passages in cell culture and these changes occur at the transcriptional level while the genotype remains unchanged (74). Transcriptional analysis of low passage/pathogenic variant (passage 10) and high

passage/non-pathogenic (passage 60) variant identified 401 genes expressed uniquely in the pathogenic variant and just 11 uniquely expressed genes in the non-pathogenic variant, while 319 were commonly expressed between both variants (74). The major differences were detected in plasmid-encoded genes, regulatory factors and ATP-binding cassette (ABC) transporter-associated genes (74). The non-pathogenic variant suppressed the whole transcriptional repertoire of plasmid A (which included virulence factor genes) and transcription level differences were detected in 3 and 10 genes present on plasmid B and C (74). Analysis of putative biological gene functions revealed that the most important reduction in gene expression was in membrane transport (72%), followed by general predicted functions (64%), cell membrane and motility (61%) and adaptation and stress response (61%) genes (74). This attenuation and altered transcriptional profile between variants could be due to the abundance of nutrients in cell culture and the lack of pressures found in host intestinal environment.

The obligate intracellular nature of *L. intracellularis* is likely an evolutionary adaptation to the microbiological and biochemical environment in the small intestine that provides protection against the influence of host immune system, commensal bacteria in the gut or influence and competition with other enteric pathogens. *L. intracellularis* may have adapted to intracellular life in the intestine by not reducing sulfates and the production of hydrogen sulfide, which is toxic to host cells, such as performed by *Desulfovibrio species* (1). A microaerophilic environment is required to grow *L. intracellularis in vitro* in different cell lines which highlights how well the bacteria have evolved to adapt to life in the porcine ileum, where oxygen tension is 5 to 10% (75). Genes that regulate pH homeostasis such as glutamate decarboxylase (GAD) system and the F_0F_1-ATPase operon have been detected in a *L. intracellularis in vitro* infection (73). The proteins produced by these systems play an important role in protecting intestinal pathogenic bacteria

against acid stress experienced in the gastric environment (76). *L. intracellularis* are well equipped to survive in the vastly different environments of the pig digestive tract and to arrive and establish infection in the small intestine crypts where pH and biochemical conditions are suitable for infection of enterocytes.

The bacterium has an affinity towards immature, undifferentiated enterocytes for invasion and growth. The conditions required for bacterial attachment and mechanism of entry into the enterocytes are not clear yet (3). An *in vitro* study of *L. intracellularis* entry into rats IEC-18 and porcine IPEC-J2 cells using electron microscopy showed that the bacteria were closely associated with the mammalian cell membrane in the first three hours of incubation (Fig. 1.3, A) (77). No specific contact structures were detected and no projecting structures were observed in uninfected control cells (77). When bacteria and mammalian cells were centrifuged together, a zone of electron-dense material known as a cap junction was formed between bacteria and cells (Fig. 1.3, B) (77). Between 3 and 24 hours post-incubation, *L. intracellularis* were internalized within membrane-bound endocytic vacuoles with only one vacuole per cell observed (Fig. 1.3, C) (77). No multiplication of *L. intracellularis* were observed in those vacuoles, and they were often found in close proximity with the mitochondria during this time (77). Also, between 3 and 24 hours post-incubation, some *L. intracellularis* were detected free in the cytoplasm and empty vacuoles could be observed which implies that they escaped the endocytic vacuoles (Fig. 1.3, D) (77). Between 2 and 6 dpi, numerous free dividing bacteria were present in the cytoplasm with no bacteria in vacuoles (77). Groups of bacteria were found adjacent to the nucleus and heavily infected cells had numerous bacteria in proximity or in contact with mitochondria and endoplasmic reticulum (77).



(McOrist et al 1995, Research in Veterinary Science 1995, 59, 255-260)

Figure 1.4 Transmission electron micrographs of thin sections of IEC-18 cells after inoculation with *L. intracellularis*. (A) Association of bacteria with cell, three hours after inoculation, x 52,000; (B) Formation of cap junctions between cell and bacteria centrifuged on to cells, 10 minutes after inoculation, x 52,000;(C) Bacterium internalized within membrane-bound vacuole, 10 minutes after inoculation, x 45,000; (D) Internalised bacteria associated with coated pit (arrow) and vesicles, 24 hours after inoculation, x 32,000.

The ability of *L. intracellularis* to escape endocytic vacuoles and to reside and multiply in the cytoplasm is speculated to require expression of genes included in operon related to the *Salmonella* pathogenicity island 2 (SPI2) (78). The SPI2 genes play an important role in *Salmonella* survival and proliferation in infected macrophages by interfering with trafficking of

transferrin receptor and inhibiting the fusion of *Salmonella*-containing phagosomes with lysosomes and endosomes (79). The presence of *L. intracellularis* were reported by using fluorescence in situ hybridization (FISH) in *lamina propria* macrophages (12) although it was not clear whether they could persist and multiply in macrophages or whether they were phagocyted and would be subsequently processed by the macrophages. During *L. intracellularis* infection of enterocytes, expression of SPI2 operon-like genes may help bacteria to escape the vacuole. Once in the cytoplasm, they remain in close proximity to the mitochondria and ribosomes where they can obtain energy and nutrients for persistence and multiplication inside the cell. The presence of genes encoding ATP/ADP translocase in *L. intracellularis* genome confirms that the bacterium is dependent on host cells energy sources, which may explain why *L. intracellularis* thrive in close proximity to mitochondria (80). The *L. intracellularis* ATP/ADP translocase plays an important role in the exchange of bacterial ADP with ATP from the host cells and it shares 47% sequence similarity with the Rickettsia and Chlamydia ATP/ADP translocases (80). This 'energy parasitism' is essential for the survival of obligate intracellular bacteria inside eukaryotic cells.

The RNA sequence analysis and comparative transcriptional analysis of pathogenic and non-pathogenic *L. intracellularis* genome revealed the expression of genes related to oxidative protection mechanism (71). Also, Cu-Zn superoxide dismutase (*sodC*) genes, rubrerythrin-rubredoxin (*rubyY-rubA*) operon and dioxygenase genes (dioxygenases related to 2-nitropropane dioxygenase) were highly expressed by *L. intracellularis* in the enterocytes cytoplasm in study using laser microdissection with RNA-seq analysis (71, 78). These genes code for proteins that allow the bacteria to cope with oxidative stress by neutralizing reactive oxygen species inside the endosome (81).

Copper is a metal used by mammalian cells during the process of oxygen reduction by cytochrome C oxidase which is essential for ATP production (82). Controlling copper levels is one of the innate immune mechanisms by which mammalian cells defend themselves from intracellular pathogens (78, 82). Infected cells have a high upregulation of high-affinity copper uptake genes on the apical membrane (78) which may prove toxic to the invading pathogen. However, *L. intracellularis* has developed ways to use the host cell's energy and defense mechanisms to its own advantage and to thrive in the cytoplasmic environment despite high copper concentration. *L. intracellularis* expresses Cu-Zu superoxide dismutase to take up intracellular copper to promote survival in the intracellular environment.

Other studies show that many important genes involved in host cell nutrient acquisition such as genes responsible for uptake of Vitamin B12, bile acid, glucose, amino acids, disaccharide, and lipids are down-regulated after infection (78). Further, genes that code for proteins responsible for electrolyte secretion on the apical membrane were shown to be highly downregulated. In contrast, genes encoding glucose transporter 1 (also known as solute carrier family 2, glucose transporter member 1) and the high-affinity copper uptake protein (CTR1) involved in copper absorption were highly upregulated. Altogether, these changes in gene expression may produce an excessive concentration of nutrients and electrolytes in the intestinal lumen which may correspond to induction of osmotic diarrhea, the main symptom of PE in pigs (78).

1.2.3 Induction of proliferation and inhibition of differentiation of infected enterocytes

L. intracellularis has evolved to exploit the intracellular environment and to rely on host cell multiplication for its own propagation. Proliferating cells offer a suitable microenvironment for the bacteria to acquire the necessary nutrients and energy for replication. Upon entry into the undifferentiated small intestine crypt cells, *L. intracellularis* thrive and, using a mechanism that is not yet fully understood, induces cellular proliferation and prevents differentiation of immature crypt cells into different subsets. To elucidate the mechanism behind proliferation of invaded immature cells, several studies with diverse approaches including microarray analysis (83), laser microdissection with RNAseq (78) and immunofluorescence (IF) coupled with quantitative reverse transcriptase PCR (RTqPCR) (84), were undertaken.

Microarray analysis was used to examine the effect of *L. intracellularis* infection on gene expression of a mouse fibroblast (McCoy) cell line (83). Mouse DNA microarrays with 44,498 genes targets were probed with cDNA from infected McCoy cells led to identification of over 40 genes with ≥ 4-fold change in expression between 0 and 3 dpi (83). This study showed that *L. intracellularis* infection induced transcription of genes important in the regulation of the cell cycle or cell differentiation genes, such as *usp18, Hr, Elavl2* and *Slfn2* (83) as well as genes that play a role in the host cell "alarm response" to invading microbes, such as interferon-related genes *Isgf3g* and *Igtp* (83). Unfortunately, murine fibroblasts infected with *L. intracellularis* may not accurately represent infection, propagation, and alteration of the transcriptome in porcine intestinal epithelial cells. Fibroblast may have more a pronounced anti-bacteria "alarm" response due to their role in antigen-processing and immune response. Further, McCoy cells do not express genes coding for intestinal enzymes or other factors that influence proliferation and differentiation in enterocytes,

in vivo (83). *L. intracellularis* proliferates within immature intestinal cells *in vivo* thus proper insight into the mechanism that induces this phenomenon can more accurately be achieved by analyzing gene or protein expression changes in these cells, *in vivo*.

A study from Vannucci et al 2013 (78) applied laser microdissection coupled with RNA sequence (RNA seq) analysis on porcine enterocytes infected with *L. intracellularis* to obtain detailed information on gene expression in targeted cells. Laser microdissection allows precise selection of infected enterocytes in the small intestine. This study identified induced expression of genes associated with cell cycle regulation such as the Rho family of genes (RhoA, RhoB and Rho GTPase) (78). The Rho family of proteins influence the G_1-checkpoint of the cell cycle when cell receive signals to enter proliferative stage (78). If these signals are absent or inhibited, the cell enters a non-proliferative stage (G_0) and therefore induction of these genes may contribute to cellular proliferation (85). Rho proteins could also be activated by bacterial toxins known as cyclomodulins (85). Although cyclomodulins in *L. intracellularis* have not been established, significant activation of the Rho family of genes in host cells and expression of hypothetical proteins in these bacteria suggest that yet uncharacterized cyclomodulins may be responsible for induction of proliferation (78). More functional studies focusing on the changes of expression of these genes and their corresponding proteins are needed to reveal the process behind the induction of the Rho family of genes and the involvement of hypothetical bacterial proteins.

To be able to precisely monitor all stages in *L. intracellularis* infection, Huan et al. 2017 collected samples of pig ileum at 3, 7, 14, 21 and 28 dpi (84). Limited *L. intracellularis* staining were observed in crypts at 3 and 28 dpi and the strongest staining of bacteria was observed at 7 and 14 dpi (84). These results correlate well with previous reports that indicated that the peak of *L. intracellularis* infection and characteristic cell alterations were observed 14 dpi (13, 10). The

depletion of goblet cells is an important hallmark of *L. intracellularis* infection and is an important step during pathogenesis. Goblet cells secrete mucins and form the first line of defense against intestinal bacteria. MUC2 plays an important role in maintaining the mucosal integrity and depletion of *Muc2* in knockout mice leads to improper colonic mucosal layer formation and subsequent increased permeability that aids to bacterial adhesion to the epithelial cells and colonic infection (86). By using immunofluorescence to stain for MUC2 (a marker for goblet cells) they obtained evidence that goblet cells were absent at 14 dpi but were present at 3, 7 and at 28 dpi (84). Further, transcript levels of *MUC2* were significantly downregulated at 14 dpi compared to the other points and uninfected crypts (84). These results indicate that depletion of goblet cells occurs between 7 and 14 dpi and that their presence is restored at 28 dpi (84). Decreased levels of MUC2 together with *TFF2, TFF3, RETNLB* (genes encoding the proteins produced only by goblet cells) and CLDN15 were decreased between 3- and 9- fold at 14 dpi which correlates well with previous studies (87) and strengthens the claim that disturbance in mucosal integrity and loss of goblet cells are due to *L. intracellularis* infection of intestinal crypt cells (88). Also, this study reported down-regulation of *ATOH1* at the 14 dpi and implies that Notch signaling during infection leads to inhibition of secretory lineage cells (84).

The intestinal epithelium is segmented into crypts and villi with the distinctive difference in cell types. The crypts are comprised of proliferative cells, divided into intestinal stem cells and their progeny, transit amplifying (TA) cells which remain in the crypt around two days, dividing 4-5 times before they undergo differentiation into specialized differentiated cells (89). The villi are comprised of differentiated cells which include: absorptive enterocytes, mucus-secreting goblet cells and hormone-secreting endocrine cells (89). TA are differentiated cells that move up along villi and after three days they undergo apoptosis and are released into the intestinal lumen (90). In

contrast, Paneth cells which are important for innate immunity and antibacterial defense, are differentiated in crypts and migrate downward instead of upward (89).

Cellular proliferation and differentiation are finely balanced processes regulated by different biological factors within and outside the cells. The β-catenin/Wnt pathway plays an important role in regulating epithelial cell proliferation. In a resting state, β-catenin is bound by adenomatous polyposis coli protein (APC protein), casein kinase I (CKI), Glycogen Synthase Kinase 3 (GSK3), and axin protein complex which either promotes β-catenin degradation through the process of phosphorylation or it is bound to E-cadherin and form the membrane complex (91). The β-catenin/Wnt pathway is activated by the binding of Wnt to Frizzled and low-density lipoprotein, which initiates degradation of a protein complex that inhibits β-catenin (89). Ultimately, this leads to increased levels of β-catenin in the cell (89). β-catenin is now free to enter the nucleus where it binds to the TCF transcription factor which in turn leads to expression of Wnt targeted genes (89). Induced expression of these genes induces proliferation of cells and is an important physiological process for renewal of cells in the intestine. Mutations in the Wnt signaling pathway leads to accumulation of β-catenin and transcriptional activation of WNT/TCF4 targeted genes which begin the transformation of intestinal epithelial cells (92). These mutations are responsible for intestinal hyperplasia and developing colorectal cancer (93).

The Notch pathway is another imported cellular pathway in the intestine that regulates intestinal stem cell survival, drives maturation of TA cells into absorptive enterocytes and suppresses the secretory lineage but no mutations in Notch signaling were reported in intestinal tumorigenesis (89, 94). Mutations or disturbance in β-catenin/Wnt and Notch signaling pathways lead to hyperplasia and secretory cell dysplasia of intestinal cell epithelium which is also characteristic for *L. intercellularis* infection. Thus Huan et al 2017 explored the changes in β-

catenin/Wnt and Notch signaling pathways during *L. intracellularis* infection of intestinal epithelial cells.

In a recent study, Huan et al 2017 used IF coupled with RT-PCR to gain insight into biological mechanisms behind induced proliferation and inhibition of differentiation in ileal crypt intestinal cells infected with *L. intracellularis* (84). The authors speculated that *L. intracellularis* infection of crypt cells must alter the β-catenin/WNT signaling which is necessary for normal intestinal stem cell proliferation and self-renewal and at the same time impacts the Notch signaling which is essential regulator of differentiation of transit amplifying cells (TA) to secretory and absorptive cell lineage (84).

To have a better understanding of the proliferative effect of *L. intracellularis* on crypt cells, Huan et al 2017 applied a more targeted approach to explore gene changes in expression of genes known to be involved in intestinal stem cell proliferation and differentiation (84). Their targets were *c-MYC,* which encodes a "master regulator" oncogene responsible for control of multiple stages in cell growth and metabolism, and *ASCL2* which encodes intestinal stem cell marker necessary for proliferation (84). Down-regulation of these two genes after 7 dpi onwards implies that intestinal stem cells are not the ones that proliferate but rather the proliferating cells are TA cells and/or absorptive progenitor cells (84). Interestingly, 21 dpi onward *LGR5*, a specific marker for intestinal stem cells, was down-regulated compared to uninfected cells from 21 dpi onward (84), which opens the possibility for further exploring subtle specificity in *L. intracellularis* affinity towards crypt cell subtypes.

Analysis by IF showed that cells stained as positive for β-catenin in the cell membrane and cytoplasm of uninfected and *L. intracellularis* infected crypts, respectively, at 3 and 28 dpi (84).

As infection progressed, β-catenin staining intensified in infected crypts and it was highest corresponding to the peak of infection at 14 dpi (84). Also, infected cells showed increased staining for SOX9 and AXIN2 staining, negative regulators of β-catenin/WNT pathway, between 7 and 14 dpi with significantly higher intensity in the lower than upper crypts (84). To further explore the effects of *L. intracellularis* on β-catenin/WNT pathway, expression of the Notch-1 receptor (NICD1) was examined by IF in infected and uninfected crypts (84). An increased NICD1 staining was observed in infected crypts at the peak of infection which indicates that Notch-1 signaling pathway is active (84). Active Notch-1 signaling inhibits atonol homolog 1 (ATOH1) expression which leads to differentiation of TA progenitor cells into absorptive cells (instead of secretory lineage) (84). The activation of the Notch-1 pathway was supported by down-regulation of *ATOH1* transcripts and low mRNA levels of β-catenin/WNT signaling ligand WNT3 which together implies that β-catenin/WNT pathway was altered (84). This result correlates well with research in mouse colons which showed that induction of β-catenin expression led to expression of genes that induced expansion of the LGR5+ intestinal stem cells and reduced TA progenitor cell proliferation. Further, they showed that down-regulation of β-catenin/WNT pathway enhanced proliferation of progenitor cells but not intestinal stem cells (95, 96). Huan et al 2017 found an increase in expression of Ki67, SOX9 and NICD1 in crypts at the peak of infection which implies multiplication of TA and/or absorptive progenitor cell population. This research may lead to determining differentiation state of epithelial cells which are susceptible to *L. intracellularis* infection.

Hyperplasia of the small intestine mucosa, proliferation of the immature enterocytes, and depletion of goblet cells are an important characteristic of lesions induced by *L. intracellularis in vivo*. Current knowledge on the mechanism by which bacteria induces these changes suggest a

very complex mechanism and interplay between bacteria and host intestinal cells. The bacteria have developed molecular mechanisms to influence the molecular base of the cell cycle and impact the proliferation and differentiation of intestinal cells. Molecular signaling pathways such as Rho family genes (RhoA, RhoB and Rho GTPase) (which act on cell cycle), β-catenin/WNT and Notch-1 are altered and suspected to be the cause of altered cell proliferation and depletion of goblet cells. The proliferation of murine colon enterocytes have been described in *C. rodentium* infection but in this case, β-catenin/WNT and Notch-1 pathways were up-regulated at the peak of infection (97). Although histologically, proliferative lesions are similar histologically, molecular pathways and possible bacterial mechanisms are different between *C. rodentium* and *L. intracellularis*. These findings indicate a unique model by which *L. intracellularis* influence the intestinal cell proliferation and depletion of goblet cells. The exact mechanism by which *L. intracellularis* induces those cellular changes are still not known and further research is needed to elucidate first bacterial genes and proteins involved and then exact host cell signaling pathways that lead to observed lesions.

1.2.4 Isolation and maintenance, *in vivo*

L. intracellularis resides in the apical cytoplasm of the immature crypt enterocytes (13). Successful isolation and propagation of *L. intracellularis* were achieved in 1993 by Dr. Lawson and his group after almost 30 years of research (4). Isolation of this intracellular pathogen from infected intestines requires quick processing, careful manipulation, and adequate tissue condition to maintain viability. Because *L. intracellularis* is resistant to vancomycin and neomycin, culturing in the presence of these antibiotics prevents the growth of many other intestinal bacteria. The

isolation process begins with longitudinal dissection of the small intestine and careful removal of intestinal content followed by scraping of the mucosa, followed by homogenization in the presence of 1% trypsin in PBS for 35 min at 37℃ (4). Ten ml of trypsinized and homogenized mucosa is diluted four times in Dulbecco's modified Eagle medium with 10% fetal calf serum and then ground in a tissue grinder and filtered through a 200-mesh stainless-steel filter, through a glass fibre filter, and through 1.2-, 0.8-, and 0.65-, μm-pore size membrane filters (4). From our experience with isolating *L. intracellularis*, including 40-, 10- and 5-μm pore size filters is advisable to gradually decrease the viscosity of suspension and to achieve better filtering through smaller pore filters. To achieve additional purification, 4 ml of the *L. intracellularis* filtrate can be loaded onto 30 ml of a 45% Percoll gradient and centrifuged 20000 x g for 45 min (98). After centrifugation bacteria is present in the lower band of the gradient which is collected and washed with 12 ml PBS at 8000 x g for 20 min (98). The supernatant is discarded and pellet is suspended in freezing medium. Filtered isolate is resuspended in dimethyl sulfoxide (DMSO) to a final volume of 10% at room temperature, and aliquots are frozen at -70°C (4). Instead of using DMSO, filtrates could be prepared for freezing by diluting with sucrose potassium glutamate solution with 10% FCS (4).

L. intracellularis can be cultivated and maintained *in vitro* in various mammalian cell lines such as rat small intestinal (IEC18 cells), McCoy cells, human fetal intestine cells, human intestinal cells (INT 407 cells), rat colonic adenocarcinoma cells, (CRL1677 cells), and pig kidney (PK-15) cells (4). *L. intracellularis* need a microaerophilic environment and dividing eukaryotic cells for growth. The conventional method for isolation and cultivation is in a static adherent monolayer cell culture with incubation in humidified tri-gas incubator with 83.2% nitrogen, 8.8% carbon dioxide and 8% oxygen at 37℃ (4). An alternative method for cultivation is in chambers, (‘original

space bags or Zip lock bags') which are inflated with a mixture of 10% hydrogen, 10% CO_2 and 80% nitrogen gas (99). To cultivate *L. intracellularis* at commercial scale for live attenuated vaccine production, bacteria are grown in automatized bioreactors with stirring and recording of temperature and gas mixture (100). Bacteria isolated from the infected intestine is grown in DMEM with 10% FCS, plus neomycin or gentamicin (50 μg/ml), vancomycin (100 μg/ml) and amphotericin B (2.0 μg/ml) to suppress growth of contaminating intestinal bacteria (4). Medium is changed every 2 to 3 days with DMEM plus 5% FCS and antibiotics. Cells are trypsinized at weekly intervals and seeded at 0.5 x 10^5/ml into fresh T-75 tissue flasks (4). After successful several propagation in cells, bacteria can be propagated in medium without antibiotics.

Due to this specific *in vitro* growth requirements and the complexity of isolation of the bacteria from infected intestines, *L. intracellularis* are very difficult to cultivate and maintain in laboratories. These difficulties have led to a relative lack of information regarding virulence factors such as cell surface proteins and/or proteins responsible for the *L. intracellularis* pathogenesis.

1.2.5 Immunogenic antigens of *L. intracellularis*

L. intracellularis antigenic properties have still not been fully elucidated. Currently, it is known that *L. intracellularis* has 25 to 27 visible protein bands after electrophoresis on a one dimensional SDS-PAGE, from which 22 are conserved in six different isolates (1). The protein masses of the major bands are 53 kDa, 42 kDa, 37kda and 30 kDa (1). The serum polyclonal 1999PAb antibody targets five outer membrane proteins (OMPs) with masses of 77, 69, 54, 42 and 36 kDa while the monoclonal 2001MAb targets OMP of 77 kDa, and monoclonal IG4 targets a protein of 18 kDa (101). Other select proteins that constitute the outer membrane have been

detected and characterized such as LsaA (27 kDa), which is involved in cell attachment and invasion (102) and LatA autotransporter protein (72 kDa) (103).

LsaA is predicted to have role in cell attachment and invasion and is expressed during *in-vitro* infection of IEC-18 cells and also in infected enterocytes from ileum tissue (102). This antigen is recognized by the monoclonal antibody VPM53 using IHC detection in both IEC-18 cells and tissue samples (102). Based on its similarity to proteins of other Gram-negative bacteria, LsaA is believed to have a role in pathogenicity during infection. Further studies are needed to characterize its function and cellular networks.

The outer membrane protein LatA, LI0649 belonging to the family of autotransporter proteins was detected using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (103). The predicted LatA molecular mass was 91.9 kDa but a 72 kDa form of LatA was observed on SDS-PAGE gels which implies that protein cleavage occurred (103). This gene was cloned and the purified protein was recognized by the immune serum from pigs infected with *L. intracellularis* indicating that is antigenic (103). This protein is highly specific to *L. intracellularis* and it can be exploited for development of diagnostic tests for PE.

The same group applied shotgun proteomics and identified 19 proteins in two *L. intracellularis* isolates (104). Two proteins, LI0841 and LI0902 were predicted as outer membrane-associated proteins (104). The rLI0841 protein was identified as a putative invasin with sequence similarity to proteins that promote invasion. The rLI0902 protein was predicted to be involved in protein-protein interactions. Both recombinant proteins were recognized by serum antibodies from infected pigs (104). Recognition of these two recombinant proteins by pig immune

sera indicate that they are expressed and available to host immune recognition thus making them potentially good diagnostic tools or antigens for a subunit vaccine.

L. intracellularis, like all Gram-negative bacteria, have lipopolysaccharide (LPS) in the outer membrane which has been used as an antigen target for ELISA antibody detection in pig serum (27). LPS based ELISA are not ideal diagnostic tools due to the high cost of LPS antigen extraction and differences in background LPS reactivity among animals (27).

Analysis of 20 genes highly expressed by *L. intracellularis* when it resides in the enterocyte cytoplasm led to identification of 7 unknown genes whose corresponding proteins are part of the outer membrane surface (78). These hypothetical OMPs could play roles in attachment and invasion of host cells and thus might be recognized by the immune system of the host. Further investigation into their immunogenicity is needed before it can be determined if this proteins are critically required for *L. intracellularis* infection of enterocytes.

An antigenic and functional study was performed on the flagellin-like protein (LI0570) that was recently identified using *in silico* computational approaches (105). The recombinant LI0570 protein was detected in Western blots using mouse anti- *L. intracellularis* hyperimmune sera indicating that this protein is immunogenic (105). Further, HEK-Blue cells incubated with the rLI0570 protein led to stimulation of TLR-5 signaling and IL-8 production which implies its function in innate immune cell, *in-vivo* (105). Due to the potential immune-stimulatory effect of rLI0570, this recombinant protein have a dual role as antigen and adjuvant and may be an excellent candidate to develop recombinant subunit vaccine.

1.3 IMMUNE RESPONSE TO INFECTION WITH *L. INTRACELLULARIS*

1.3.1 Characteristics of the mucosal immune system in the intestine

The gastrointestinal system is a tubular structure that has evolved to have numerous important functions including food reception and storage, propulsive transport, digestion, absorption, barrier properties, and elimination/evacuation (106). This tubular structure is lined with a continuous layer of epithelial cells positioned on a basement membrane that separates epithelial cells and loose connective tissue called the *lamina propria*. The *lamina propria* is comprised of blood and lymphatic vessels and numerous immune cells organized in mucosal-associated lymphoid tissue (MALT) or dispersed freely in connective tissue. Beneath the *lamina propria* lies the submucosa which is the layer of dense connective tissue that binds mucosa to the smooth muscle layer which is responsible for peristaltic movements. Intestines are segmented based on the specific anatomical structures and their role. The small intestine consist of duodenum, jejunum, and ileum, which together have a length between 5.5 m to 7 m in humans and 15 m to 22 m in pigs (107). The human and pig small intestinal anatomy of the small intestine in humans and pigs is very similar, with the ratio of body weight to intestine length approximately 10:1 in both species, making the pig a good model for human intestinal disease investigation (107). The surface area of human intestine has been estimated to be between 300 (108) and 400 m^2 (109) (often compared to the size of a tennis court) but recent studies showed that the estimated surface area is much smaller, around 32 m^2 (106). This enormous surface area is due to the presence of small intestinal villi that are lined with absorptive and secretory enterocytes which themselves have microvilli (cellular membrane protrusions) on their surface. The main role of the intestine is the

digestion of food and absorption of nutrients but it also acts as an important barrier between the outside world and the sterile environment within the body. The lumen of the intestine is densely populated with bacteria which are often referred as commensal bacteria. The terminology is designated for two organisms living side by side wherein one organism benefits from the other and one organism is unaffected. These luminal bacteria can by defined more precise as mutualist due to the fact that both host and bacteria benefit from their relationship (110). The estimated number of bacteria in the small and large intestine is approximately 10^{14} which is 10 times more than the total number of cells in the human body (111). They play an important role in nutrient digestion, especially for degradation of mammalian dietary components that are not readily digestible by the host. The other important role that has been investigated more in recent years is a role in the regulation of the immune system of the mammalian host. Recently, evidence points to important cross-talk taking place between commensal bacteria and hosts epithelial and immune cells. Commensal bacteria promote the proper development of host immune system and provide protection against colonization by intestinal pathogenic bacteria. The sheer surface area of the intestinal mucosa and the vast number of commensal bacteria present in the lumen provide a complex environment for the intestinal immune system. The intestinal immune system has evolved to distinguish between resident commensal bacteria and pathogenic bacteria and to provide protection against invasion while maintaining the intestinal integrity and normal nutrient digestion and absorption. Thus, a major characteristic of the innate and adaptive intestinal immune system requires implementing immune tolerance to prevent excessive inflammation.

1.3.2 Intestinal innate immunity

As part of innate immune system epithelial cells and antigen presenting cells comprise the first line of defense against intestinal pathogens. The epithelial cells that line the gut wall have tight connections between adjacent cells through proteins including claudins, zonula occludens and junctional adhesion molecules, which together form tight junction complexes. Tight junction complexes prevent the movement of pathogens from the lumen to the *lamina propria*. Most mammals are born with a semipermeable mucosal epithelial layer which is crucial for nutrient acquisition and immune cell transport from mother to her offspring. Epithelial cell subtypes develop from the intestinal stem cells at the bottom of the intestinal crypts. Proliferating stem cells give rise to transit amplifying cells which differentiate in response to cellular signals into Goblet cells, absorptive enterocytes, Paneth cells, endocrine cells, and antigen-sampling microfold (M) cells. Each epithelial cell plays a specific role in immune regulation and/or maintenance of gut barrier.

The enterocytes belong to the absorptive lineage of intestinal cells responsible for absorbing and transporting nutrients from intestinal lumen to blood and lymphatic vessels in the *lamina propria*. They constitute more than 80% of all intestinal epithelial cells and form an important physical and chemical barrier that separate mammalian cells from microorganisms present in the intestinal lumen (89). They play an important role in maintaining immune gut homeostasis and they have mechanisms to detect and react to stimuli from various microorganisms in the intestinal lumen. These signals determine the type and magnitude of immune response and are crucial for maintaining optimal functioning of the intestinal immune system.

Absorptive enterocytes express pattern recognition receptors (PRRs that recognize specific conserved molecular structures produced by prokaryotes called pathogen-associated molecular patterns (PAMPs). PAMPs are conserved among prokaryotes and they include LPS, peptidoglycan and flagella from bacterial cells (112) and viral and bacterial nucleic acids (113). PAMPs bind to PRRs to initiate signaling cascades that regulate specific transcription of genes involved in immune defense or inflammation (112).

The absorptive enterocytes express Toll-like receptors (TLRs) and cytoplasmic Nucleotide-binding ologomerization domain (NOD)-like receptors (NLRs). The TLRs are membrane-bound glycoproteins that recognize PAMPs via leucine-rich repeats (114). Twelve mice, ten human (112) and ten porcine TLRs have been described to date. They are expressed on innate immune cells such as macrophages, and neutrophils (115, 116), but intestinal epithelial cells have also been found to express TLR 1 to TLR 9 (116). TLR 3, 7, 8 and 9 were originally reported as localized within endosomes and TLR 1, 2, 4, 5 and 6 were characterized as exclusively expressed on the cell surface. The field has evolved to recognize that TLR localization may not be conserved across all cell-types and may change in response to stimulation, age, or disease.

The binding of TLRs to PAMPs initiates signaling cascades (the specifics of which depend on the recognized PAMP and its cellular localization) through a cytoplasmic Toll/interleukin-1 receptor (TIR) domain (115). TIR activation recruits the signaling adaptor proteins, MyD88 (Myeloid differentiation primary response 88), MAL (Myelin and lymphocyte protein), TRIF (TIR-domain-containing adapter-inducing interferon-β) and TRAM (Translocation Associated Membrane Protein) and triggers the nuclear translocation of transcription factors such as NFκB, activation protein 1 (AP-1), interferon response factor 3 (IRF3) and IRF7 (117). NFκB and AP-1 act as transcription factors to promote the expression of proinflammatory factors such as tumor

necrosis factor-α alpha (TNFα), interleukin IL-1 and IL-8 (118). The transcription factors IRF3 and IRF7 are responsible for the production of type I interferons, IFNα and IFNβ.

There are region-specific differences in the localization patterns of the TLRs in the enterocytes in the small and large intestine. Colonic epithelial cells express TLRs 1-9 but only TLR1-5 and TLR9 have been detected in intestinal epithelial cells of the human small intestine and they differ in the level of expression and cell localization (119). Expression of TLR2 and TLR4 on ileal crypt enterocytes in the human fetus were localized to the basolateral surface (120). TLR2 was expressed in both the follicle-associated epithelium (FAE) and the IECs of the villi and crypts in mice (121). In pigs, TLR localization changes with age. In pre-suckling piglets, TLR2 and TLR9 expression was detected in the cytoplasm and on the apical surface of M cells but not on absorptive enterocytes (122) whereas TLR9 was highly expressed on the surface of enterocytes and FAE including M cells in adult pigs (123).

Uncontrolled inflammation in the intestinal mucosa is detrimental to gut homeostasis and prolonged inflammation such as in case of Crohn's disease or intestinal bowel disease (IBD), can lead to serious damage of the intestinal epithelium. Normal adult human colon shows low TLR2 and TLR4 expression whereas patients with active Crohn's disease (but not patients with ulcerative colitis) have high expression of TLR4 on the apical surface of colonic IECs (124). TLR5, a receptor for bacterial flagellin, is localized to the basolateral surface of enterocytes and thus can only bind to the flagellin of bacteria that have invaded the cell and traversed the gut barrier (116). By being localized away from the luminal surface, the inflammatory response is triggered only when microbe invaded through the gut barrier and not in response to commensal bacteria in the intestinal lumen.

Another large group of PRRs are the NLRs with the important role of sensing PAMPs in the host cells cytoplasm. NOD1 and NOD2 are located in the cytoplasm of intestinal epithelial cells and macrophages. NOD1 recognize muramyl tripeptides that are components of Gram-negative bacteria (125) while NOD2 is activated by a specific muramyl dipeptide common to both Gram positive and Gram negative bacteria (126). NOD1 and NOD2 binding of their ligands leads to induced transcription of proinflammatory genes through adaptor molecules RIP2 and CARD, which activate NF-κB transcription factor (127, 128). NOD1 and NOD2 are important innate immune receptors for the clearance of gastrointestinal infections caused by *Listeria monocytogenes* and *Helicobacter pylori* (129, 130).

The IECs contribute to immune protection by transporting secretory immunoglobulins across the epithelial barrier. Immunoglobulins are produced in plasma cells located in the *lamina propria.* To become secreted, dimeric IgA complexes bind the polymeric immunoglobulin receptor (pIgR) on the basolateral membrane of IECs and are then actively transcytosed to the apical surface (131). The pIgR is endoproteolytically cleaved and five extracellular Ig-like domains of the pIgR are released with IgA (two IgA bound by J chain) into the intestinal lumen (131). These pIgR cleaved components are known as the secretory component (SC) and together with IgA from the sIgA complex (131). The SC alone and as part of sIgA has mucus-binding properties, which helps to constrain bacteria in the mucus layer, stimulate biofilm formation, and prevent adhesion of bacteria to the epithelial surface (131). The SIgA antibodies in mucosal secretions are important for maintaining immune homeostasis. They limit contact of microbial and environmental antigens with the subepithelial tissue cells and blood vessels, maintain the integrity of the epithelial barrier, and influence the composition of the commensal microbiota (131). Recent data suggests that PAMPs stimulate expression of pIgR by IECs as part of a homeostatic loop in which the microbiota

enhance the production of SIgA, which in turn regulates the composition and function of the microbiota (131). Microbiota play an important role in the development of the intestinal immune system, including class switching to IgA and maturation of plasma cells (132).

Goblet cells secrete glycosylated proteins called mucins to form the mucus layer that covers the intestinal mucosa. This gel-like substance is formed by MUC2, MUC5, and MUC6, and serve as a chemical and physical barrier to the pathogens by binding to proteins to block attachment to epithelial cells (86). Beneath this secreted mucus layer is the glycocalyx which tightly adheres to the apical surface of enterocytes and forms a dense physical barrier. Membrane-bound proteins present in the glycocalyx include MUC1, MUC3A/b, MUC12, MUC13, and MUC17. The glycocalyx is almost sterile in mice with only segmented filamentous bacteria (SFB) attached to enterocytes via their filaments. In contrast, the secreted mucus layer is home to various commensal bacteria that have evolved to thrive in this environment (133). Secretion of mucus is an important factor in maintaining the homeostasis of the intestinal mucosa. Disturbance in mucus production or secretion leads to increased inflammation and destruction of the epithelial barrier, and these are hallmarks of many intestinal diseases such as inflammatory bowel disease (IBD), Crohns's disease, colorectal cancer, intestinal bacterial infections, etc. A signature trait of *L. intracellularis* infection includes increased proliferation of immature intestinal cells, reduced numbers of Goblet cells and lower expression of MUC2 (88). MUC2-deficient mice spontaneously developed colitis and inflammation-induced colorectal cancers which indicates that this protein plays an important role in regulating intestinal homeostasis (134, 135). These data indicate the indispensable role of mucin secretion from goblet cells in regulating integrity and proper functioning of the intestinal epithelial barrier. Recently it was determined that goblet cells play a role in antigen uptake and presentation to DCs in the *lamina propria* (136). Specifically, goblet cells transport low molecular

weight soluble antigens to $CD103^+$ CX_3CR1^- *lamina propria* dendritic cells (136). Upon antigen uptake, there is induced production of the non-inflammatory immunoglobulin, secretory IgA (SIgA) production, imprinting of gut homing on cognate lymphocytes, and induction of regulatory T cells (136). Thus antigen sampling through goblet cells induce immune tolerance and promotes in maintenance of gut homeostasis.

The Paneth cells play an important role in innate immunity of the intestinal mucosa by secreting α-defensins, C-type lectins, lysozyme, and phospholipase A2 in the form of granules into the intestinal lumen, which provides antimicrobial protection for epithelial cells (137). Paneth cells also secrete epidermal growth factor (EGF), WNT3 and the delta-like canonical Notch ligand 4 (Notch ligand Dll4), which are important signals for proliferating epithelial stem cells responsible for epithelial renewal (138, 139). Likely due to their role in secretion of antimicrobial peptides and proteins, they have evolved to avoid being sloughed off the villi by instead migrate downwards to the crypt bottom (89).

The enteroendocrine cells constitute only around 1% of the total intestinal cells but they play an important role in the secretion of peptide hormones (140). Enteroendocrine cells in mice and humans express TLR1, TLR2, and TLR4 which co-localize with serotonin, a marker for enteroendocrine cells (141). Induction of these pathogen-sensing receptors leads to secretion of secreted peptide hormones such as secretin, gastric inhibitory polypeptide (GIP), serotonin, somatostatin, gastrin, and cholecystokinin (CCK). These hormones are important regulators of multiple metabolic and physiological processes in secretion of intestine-like fluid and electrolytes, intestine motility, blood flow and food intake (142). For example, after stimulation of TLR5 and TLR9 with flagellin and CpG oligodeoxynucleotides (CpG ODN), respectively, mouse enteroendocrine cells induced secretion of CCK, which lead to induced of gallbladder and small

intestine contractions (143). Recognition of PAMPs by enteroendocrine cells might aid in clearance of lumen pathogens by increasing peristaltic movement of the intestine and enhancing evacuation of lumen content.

The M cells are modified epithelial cells that shuttle antigen from the lumen to the *lamina propria*. They are situated above the gut-associated lymphoid tissue (GALT) that includes isolated lymphoid follicles (ILFs), Peyer's patches in the small intestine and their equivalent in caecum and colon, and isolated lymphoid follicles (ILFs) (144, 145). The M cells constitute approximately 10% of the epithelial cells among these FAE (146), which are distinguished from other IECs by the presence of a much thinner glycocalyx, short, irregular microvilli and large openings on their surface. M cells have unique role in phagocytosis and transcytosis of gut lumen macromolecules, antigens, and pathogenic or commensal microorganisms through the epithelial barrier (144). The M cells deliver antigens across the FAE basolateral membrane to lymphocytes and heterogeneous populations of macrophages and classical DCs (147, 148). They transport antigens in endocytic vesicles and deliver them by exocytosis to DCs in the intraepithelial pocket of GALT structures but M cells do not process the antigens. While this transportation process is an important initial step in the induction of some mucosal immune responses, some pathogens such as *Salmonella* Typhimurium have evolved to exploit these cells to enter the subepithelium (144, 149). In response to a single effector molecule secreted by *S.* Typhimurium which transforms primed epithelial cells into M cells, M cells density increases during Salmonella infection to promote its colonization and invasion (149).

There are numerous immune cells located in the subepithelium that play an important role in the gut innate immune response including mast cells, macrophages, DCs, neutrophils, basophils, eosinophils, natural killer cells, and γδ T cells (114). These innate immune cells are responsible

for capturing and presenting antigen, phagocytosis, cytokine secretion and induction of adaptive immune response, which all contribute to the clearance of pathogen and restoration of immune homeostasis. The gut innate intestinal immune cells have some characteristics that distinguish them from their counterparts in other systems of the organism. They protect the intestinal tissue from pathogen invasion but at the same time recognize and tolerate a vast variety of food and commensal antigens that are constantly present in the intestinal lumen (150). The innate immune cells control inflammation and control both innate and adaptive cell function to avoid excessive inflammation and tissue damage. They are an important link between the innate and adaptive immune response but they also influence the other systems, such as blood and lymphoid system, with their secreted products.

Macrophages and DCs are related in lineage and they differentiate from a common bone marrow precursor. They migrate into different parts of the body and acquire their characteristic phenotypes under the influence of secreted products within the local microenvironment. Differentiated macrophages and DCs are involved in the defense against pathogens by antigen uptake and presentation, production of cytokines and by induction of adaptive immunity (151). Subsets of these cells such as those found in the intestine are also capable of secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGFβ) that suppress inflammation and initiate tissue repair. Within tissues, the function of macrophages and DCs overlap and they share many similar markers making it sometimes difficult to distinguish between them (114). Further complicating their identification and characterization, not all DC markers are conserved between mice, human and porcine (114). Small intestinal phagocytes in mice are distinguished by expression of specific ligands and; cluster of differentiation (CD). The $CD103^{+}$ subsets of DCs are characterized by expression of the Flt3 ligand and they express $CD11c^{high}$ MHC

class II^{high} while $CX3CR1^{+}$ subsets of DCs and macrophages are characterized by expression of CSF-1R. Macrophages express $CD11c^{low}$ MHC class II^{low} (152).

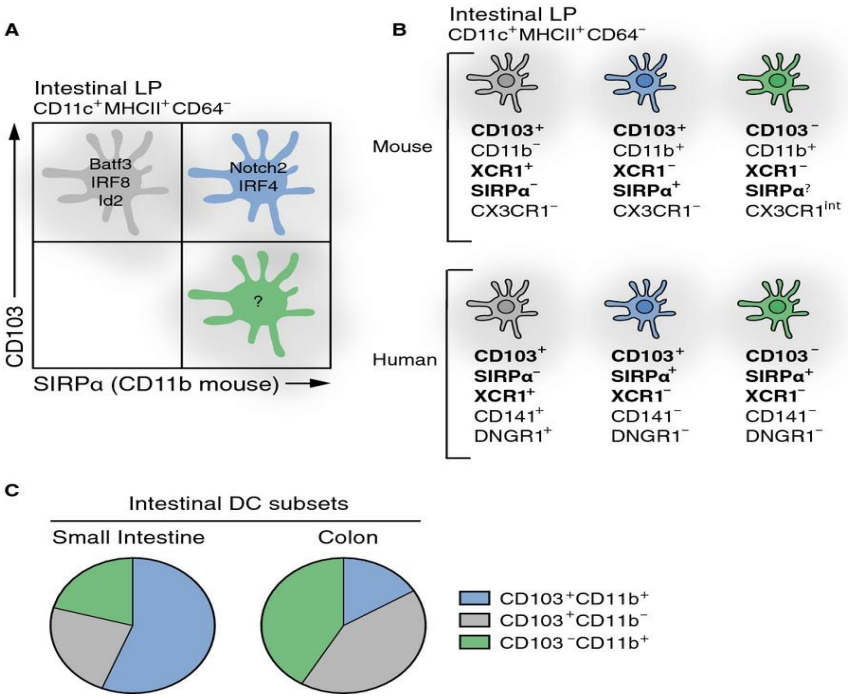
The macrophage life cycle can be divided into four distinct stages based on function and their location: differentiation, priming, activation and resolution (153). Macrophage differentiation is influenced by macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage-colony-stimulating factor (GM-CSF), retinoic acid and lipoproteins (153). After differentiation, macrophages migrate into tissues where they undergo priming influenced by the specific cytokine environment (153). Activation in the presence of IFNγ leads to "classical" activation whereas the presence of IL-4 and/or IL-13 leads to "alternative" activation (114). "Classical" activation leads to activation of a Th1-type T cell response and resistance to viral infections (151), while "alternative" activation of macrophages promote a Th2-type T cell response with increased antibody production. Once mature macrophages encounter pathogen, their PRRs will recognize their cognate PAMPs, which in turn leads to increased phagocytosis to clear the pathogen as well as secretion of pro-inflammatory cytokines. After the pathogen is cleared, macrophages enter the resolution stage, which is characterized by secretion of anti-inflammatory cytokines, clearance of cells debris and restoration of homeostasis (153). Intestinal macrophages are characterized by lower TLR4 expression than in other tissues (117), thus indicating their specialization to inhibit inflammation in response to commensal bacteria and other food antigens encountered in intestine. The anti-inflammatory cytokine IL-10 plays an important role in maintaining gut homeostasis, partially by promoting the anti-inflammatory capabilities of macrophages. Mice with mutations in the IL-10 receptor in intestinal macrophages, but not IL-10 deficient mice, developed severe spontaneous colitis (154). Macrophages can also induce tissue repair by secreting IL-13 and IL-

4, while suppressing secretion of TNF-α and IFNγ (155, 156). Tregs may be the main source of IL-10 in providing immune suppression and homeostasis (157).

Many pathogens have evolved to exploit immune suppressive trait of intestinal mucosal macrophages. *S.* Typhimurium invades macrophages and avoids their phagocytic activity by secreting virulence factors encoded in a SPI2 operon that interfere with cell trafficking (79). The SPI2 operon was also found to be expressed in *L. intracellularis*-infected enterocytes (78) and *L. intracellularis* have also been found in macrophages (158). However, whether they secrete virulence factors and survive phagocytosis has not yet been reported (3). Other enteric pathogens such as *Shigella, Listeria and Rickettsia* spp. avoid phagocytosis in macrophages by secreting lytic toxins, which help bacteria to escape from phagolysosome into the cytoplasm of the cell (159).

The intestinal DCs are professional APCs present in GALT and all over the intestinal *lamina propria* (150). They arise from the common bone marrow progenitor for macrophages and DCs called the macrophage and DC progenitor (MDP) (160). Further, MDP differentiates into common DC progenitors (CDP) which give rise to two main types of DCs: classical DCs which specialize in antigen presentation and plasmacytoid DCs (pDCs) that produce IFNα in response to viral nucleic acids (161, 162). These heterogeneous cells have different phenotypes in mouse, human and porcine and research is still ongoing to characterize the phenotype of each cell species. So far, most markers of intestinal DCs have been characterized in mice and humans (Fig.1.4) (150). $CD103^+$ $CD11b^+$ DCs are the most abundant subtype in murine small intestine *lamina propria*, but the numbers are reduced in the colon where $CD103^+CD11b^-$ and $CD103^-CD11b^+$ are prevalent (163). Porcine intestinal DCs have four main phenotypes based on the expression of CD11R1 and CD172a (164). *Lamina propria* DCs are mainly $CD172a^+CD11R1^+$, PP DCs are mainly

CD172a^{+}CD11R1^{-} in the subepithelial domes and CD172a^{-}CD11R1^{-} in the interfollicular regions (164). The Mesenteric lymph node (MLN) DCs are mostly CD172a^{-}CD11R1^{+} (164).



(Bekiaris et al 2014, Immunological Reviews Vol. 260: 86–101)

Figure 1.5. Dendritic cell subsets in the mouse and human intestinal *lamina propria*. (A) *Lamina propria* (LP) dendritic cell (DC) subsets in the mouse and human can be identified by the differential expression of CD103 and the marker CD11b in the mouse or signal regulatory protein-α (SIRPα) in human and mouse. The indicated transcription factors have been implicated in regulating the development of each of the subsets. (B) Surface markers that can be used to identify human and mouse DC subsets in the intestinal LP. Common markers expressed by both murine and human DC subsets are highlighted. (C) Pie charts indicating the relative distribution of CD103+ CD11b^{-}, CD103+ CD11b+, and CD103^{-} CD11b+ DCs in the murine small intestine and colon (data are a mean ratio from six mice).

The activation of TLRs in immature intestinal DCs promotes DC maturation trough changes in cell phenotype, upregulation of MHC II, altered cytokine secretion and antiviral

response (164). Activated DCs can recognize and engulf foreign antigens by phagocytosis, receptor-mediated endocytosis or by receptor-independent macropinocytosis and micropinocytosis. Intestinal DCs can acquire soluble antigens directly from intestinal lumen (155) or at the basolateral surface of Goblet cells, which transport low molecular soluble antigens from the lumen to the *lamina propria* (136). Recent experimental data suggest that in the murine model, intraluminal injection of fluorescently labeled ovalbumin resulted in high levels of uptake by $CXCR1^{high}$ DCs and only minor uptake from $CD103^{+}$ DCs (165). Migratory DCs express MHC-II ^{high} and consist of almost equal number of $CD103^{+}CD11b^{+}$ and $CD103^{+}CD11b^{-}$ and minor population of $CD103^{-}$ $CD11b^{+}$ (166). In turn, $CD103^{+}CD11b^{+}$ DCs acquire antigenic material from $CXCR1^{high}$ macrophages via gap junctions formed by connexion 43 protein expressed on both subsets (167). Although these data require further investigation, it highlights the plasticity of intestinal DCs and macrophages, which often have overlapping roles. Once activated DCs have taken up foreign antigen, they undergo further maturation and express the chemokine receptor CCR7 that binds specifically to chemokine ligand, CCL19 and CCL21 chemokines produced by cells within the T cell zones of MLN. Mature DCs, therefore, migrate towards the MLN in response to the CCL19 and CCL21 gradient. Migration of DCs to MLNs may be influenced by local signals. For instance, oral or intraperitoneal administration of TLR agonist in mice resulted in increased DC migration from *lamina propria* to MLN (168). Because each DC subsets express different TLRs, TLR activation by PAMPs may result in migration of distinct *lamina propria* DC subsets to MLN to influence the downstream adaptive immune response (150). Once inside the MLN, DCs present antigen to T-cells via MHCII and initiate an appropriate adaptive immune response. The intestinal DCs have an important and versatile role in the intestinal immune system as essential conveyers of signals between innate and adaptive immune response. Migrating mature DCs from

various tissues present antigens to T-cells in the lymph node directly. Low molecular weight antigens transported in soluble form by the lymph enter the subscapular sinus and go through fibroblast reticular cell conduits where they are extracted and taken up by resident follicular DCs. Other soluble antigens are taken by macrophages and DCs which carries them into the cortex. Follicular DCs transport antigen and present the antigen to MHCII activated Th2-type T cells which leads to activation of B cells. B cells can act as APCs and present protein antigens to naïve and effector T cells that have been generated by previous antigen stimulation.

Intestinal DCs induce expression of intestinal mucosal receptors α4β7 integrin and CCR9 on T- and B-cells in MLN to produce gut-homing lymphocytes (169, 170). How this occurs is not completely elucidated but it is known that retinoic acid secretion by epithelial cells is important for DCs imprinting (171). Further, intestinal DCs express high levels of retinal dehydrogenase, RALDH2, which generates retinoic acid from Vitamin A and induces the expression of α4β7 integrin and CCR9 on T- and B-cells in MLN (172). The mechanism by which the intestinal DCs (an the intermediary cells) communicate to the activated T and B cells is not yet clear but the effect is that the activated T and B cells migrate to the *lamina propria* of the intestine. Intestinal DCs can also preferentially secrete IL-10 and TGFβ to promote induction of T regulatory ($FoxP3^+$ iTregs) cells or a Th2-type immune response rather than a Th1-type response which has important implications in oral tolerance and maintenance of intestinal immune homeostasis (164). An additional mechanism to maintain intestinal immune homeostasis is through promotion of the generation of IgA-secreting B cells and imprinting of gut-homing of these B cells (170). By promoting effector cells differentiation, gut migration of activated lymphocytes from MLN and oral tolerance, intestinal DCs are essential regulators of intestinal homeostasis.

1.3.3 Adaptive immune response in the intestine

The mammalian intestinal adaptive immune system has evolved to provide antigen-specific memory responses to intestinal pathogens, while at the same time maintaining tolerance to environmental, food or commensal antigens. Innate immune cells in the intestinal mucosa provide necessary signals in response to antigens which is communicated to and impacts the cells of the adaptive immune system cells. A dominant form of adaptive immunity in the gut is humoral immunity mediated by secreted IgA antibodies, induction of protective cell-mediated adaptive immunity mediated by Th17 cells, and induction of Treg cells that promote gut homeostasis and tolerance to food antigens (117).

The PPs and lymphoglandular complexes are the primary inductive sites in the gut, but the functions of the isolated lymphoid follicles and cryptopatches are unclear (145). GALT is rich in effector T cells and it is the major site of secretion of antigen-specific IgA which is non-inflammatory (173, 174). The PPs are at the highest density in jejunum and distal ileum with species-specific quantity and distribution patterns (175, 176, 177). They have germinal centres with B lymphocytes, follicular helper T cells, macrophages and follicular DCs (FDCs) surrounded by naïve B cells. Between follicles and intestinal M cells there is a dome region which also has an abundant population of B- and T-lymphocytes, macrophages and DCs. Although PPs share similar structure and function with lymph nodes, they lack a capsule and afferent lymphatics thus the main route of antigen delivery to immune cells is through M cells (178). Antigen is delivered to the APCs through M cells which then migrate to PPs to activate cognate T cells. In turn, the activated T cells bind to cognate B cells in the germinal centres. Migrating DCs can also carry the antigen to the MLN where lymphocytes become activation and receive instructions to home to the

intestinal *lamina propria*. The site of antigen uptake by APCs and the local environment thus impacts induction of a specific adaptive immune response.

1.3.3.1 Humoral immunity in the intestine

IgA is the main mediator of specific humoral immune protection and an important regulator of the commensal population (179). IgA antibodies are produced by plasma cells that stem from activated B lymphocytes which have undergone class-switch recombination in GALT (179). As detailed in Section 1. 3. 2, IgA antibodies are transported through the epithelial cells as dimers in which two IgA are joined with the J chain and transported into the lumen via the pIgR (179). At the epithelial apical membrane, this complex is cleaved and a small segment of the J chain remains associated with the dimeric IgA (referred to as secretory IgA (sIgA)), which is released into the lumen. Once in the lumen, sIgA can bind to pathogens or toxins and prevent their interaction with intestinal epithelial cells (180). Alternatively, IgA can bind pathogens in the *lamina propria* then bind to pIgR on the basolateral surface of the IECs resulting in transport and the release of the pathogen into the lumen. Intestinal IgA antibodies comprise around approximately 70% of all antibodies produced in mammals, making it the most abundant antibody class (132). Landsverk et al. 2017 reported three subsets of small intestinal plasma cells that secrete IgA in humans including the $CD19^-$ plasma cells population can remain active for >20 years and may contribute to a life-long protection against enteric pathogens (181). Oral vaccination that target activation of antigen-specific long-lived IgA secreting plasma cells may be a feasible strategy to induce long-lasting protection against enteric pathogens while at the same time maintaining necessary immune balance in the intestinal mucosa.

Since the pioneering work of Craig and Cebra 1971, there has been a general consensus that PPs are the main anatomical sites for activation of IgA secreting plasma cells (182, 183). A more recent study from Barone et al. 2011 reported that IgA-producing plasma cells are derived from GALT germinal centres in humans and that B cell receptor engagement is important for the formation of GALT germinal centres (184). The PPs offer an adequate microenvironment for the interaction of B cells with follicular DC and local Th2-type CD4+ T cells leading to B cell activation and expression of IgM antibodies. Activated B cells then undergo B cell proliferation, class switching recombination (CSR), somatic hypermutation (SHM) and affinity maturation in the germinal centre (183).

During T-dependant B-cell activation, B cells take up antigen via the B cell receptor (BCR), which is then internalized. Antigen is presented on MHCII to T cell receptor (TCR) with co-stimulatory molecule CD40 on B cell binding with CD40L on T cell (179). In response to the immunological synapse between these cells and regional cytokines such as IL-4, IL-10 and retinoic acid, B cells become activated and cytidine deaminase (AID) expression is induced (179). High levels of AID expression were found in PP IgM^+ B cells, which suggest that AID may be a major contributor required for IgA class switching and production (185). Germinal centre CD4 T cells that are involved in the T-dependant mechanism are $Foxp3^+$ Tregs (186), T-follicular helper cells (Tfh) (187) and Th17 cells (188).

The maturation of GALT depends on the presence of commensal bacteria and their products; in germ-free mice, where IgA production is impaired although B cells are present in the *lamina propria* (111). The relationship between microbiota and immune cells in GALT is bi-directional with both host and commensal bacteria cells contributing to immune homeostasis. Further, microbiota can promote Tfh-cell generation in the germinal centre of PP and T cell

dependent IgA generation and secretion (189). The importance of this synergism of host immune cells and commensal bacteria in gut lumen was elegantly shown in a study from Kawamoto et al. 2014 (186). They reported that differentiation of Foxp3+ T cells into Tfh cells was necessary for the IgA production in mouse germinal centres and that the amount and quality of IgAs had a direct impact on the diversity and phylogenetic structure of bacterial communities (186). The microbiota can promote Tfh cells generation in the germinal centre of PP and T cell dependent IgA generation and secretion (189). Findings of Kawamoto et al. 2014 showed that balanced commensal populations induced activation of Foxp3+ T cells and IgAs production which together controled diversification of stimulatory bacterial species in the lumen (186). This cross-talk between commensal bacteria and host immune cells provides a self-regulatory loop that maintains important immune balance and health in the intestine (190). It is an interesting biological mechanism that does not seek to eliminate existing bacteria to achieve sterile environment, in contrast it evolved to maintain coexistence of microbiota and epithelial and immune cells.

The T-cell-independent activation of IgA producing plasma cells depends on the local production of host cells cytokines and membrane components, commensal bacterial signals and nutritional chemicals from intestinal lumen (178). TGFβ, a cytokine produced by multiple cells in the intestinal mucosa, is an important factor for IgA isotype switching. The intestinal epithelial cells, DCs and T cells produce TGFβ but it is currently not clear which cell type is the main source of TGFβ, *in vivo* (179). The importance of TGFβ in CSR was demonstrated in *in vivo* studies where mice deficient for the TGFβ receptor, TβRII, had almost complete loss of IgA (191). The effect of TGFβ on IgA isotype switching is aided by IL-2, IL-4, IL-5, IL-6 and IL-10 secretion from different intestinal cell populations (132). Further, two cytokines important in regulating CSR are B-cell activating factor of TNF family (BAFF) and A proliferation-inducing ligand (APRIL) (132).

APRIL is secreted from epithelial cells, DCs, monocytes, macrophages and activated T cells while BAFF is secreted from epithelial cells, DCs and monocytes (132). The B cells express receptors for these cytokines and when bound by them, initiate CSR and maturation into IgA-secreting plasma cells. The DCs activated by microbial antigens produce nitric oxide which stimulates expression of TβRII and receptors for APRIL and BAFF on B cells leading to increased CSR (192). Commensal bacteria also can stimulate IgA responses indirectly through activation of TLRs on epithelial and DCs or directly through their fermentation products in the intestinal lumen. Commensal bacteria-derived short acid fat acids (SCFA) were reported to influence antibody responses, *in vitro* and *in vivo* (193). SCFA production by commensal bacteria depends on the amount of dietary fibers in animal food. Mice that had low SCFA production due to low dietary fiber intake or mice that lacked commensal bacteria had abrogated homeostatic and pathogen-specific antibody responses (193). The addition of dietary fibers and SCFAs increased intestinal IgA and IgG responses and promoted pathogen-specific response in case of *Citrobacter rodentium* infection (193). The number of IgA^+ cells in both small and large intestines were increased in mice fed dietary fibers (194). The results from this study shed light on the complex relationship between nutritional composition, commensal bacteria and host immune system, which all contribute to maintaining immune homeostasis and protection against enteric pathogens. Although this study used a mouse model, the results from this study could be extrapolated with confidence to humans or pigs due to the conserved biological functions of SCFA across animal species (195).

The importance of dietary signals in the induction of IgA production is further emphasized by the role of retinoic acid in intestinal immune homeostasis. Retinoic acid is a metabolite of dietary vitamin A and is produced by intestinal epithelial and DCs. DCs generate retinoic acid from vitamin A due to expression of retinal dehydrogenase, RALDH2, and retinoic acid secreted

by epithelial cells influence DCs gut imprinting (171). Retinoic acid produced by DCs induces the expression of α4β7 integrin and CCR9 on T and B cells in MLN (172). *In vitro* assays showed that retinoic acid induces IgA class switching and IgA production (170) while *in vivo* experiments showed that mice with B cells unresponsive to retinoic acid had significantly less IgA^+ B cells in germinal centres PPs and reduced IgA^+ plasma cells in the small intestine (196). Retinoic acid can work in concert with IL-6 produced by DCs and to induce the generation and homing of IgA producing B cells from MLN to the gut (170). Even when administered as a supplement in a subcutaneous (s.c.) vaccine to mice, the presence of retinoic acid-induced immune responses in the small intestine by promoted the gut-homing capacity on T-cells and IgA+ plasma cells in inguinal LNs (197). Finally, s.c. immunization with retinoic acid in mice induced a strong immune response in the small intestine that protected mice from cholera toxin-induced diarrhea and diminished bacterial loads in PPs after oral infection with Salmonella (197). Thus, inclusion of retinoic acid as a vaccine component induced a specific immune response (i.e. secretion of antigen-specific IgA and induced homing of lymphocytes to the small intestine) and may be an effective new vaccine strategies against enteric pathogens or a new tool for modulating the immune response in case of auto-immune diseases in humans (197). Further research is needed to determine the immune properties induced by retinoic acid in different animal species, as well as the dose needed for a safe and effective vaccine.

Activated intestinal plasma cells play important functions in maintaining gut homeostasis and protecting against infections mediated by secretion of IgA and cytokines. Secreted IgA protects mucosa by the immune exclusion of antigen/pathogen, by regulating antigen/pathogen transport and uptake and providing regulation via binding to IgA receptors (198). Due to its high degree of glycosylation, IgAs are protected from rapid cleavage which allows to trap antigens in

the mucus layer for a relatively long period of time (199). For example, protection against cholera toxin (CT), a product of *Vibrio cholerae* is mediated primarily by sIgA antibodies that block toxin attachment to the epithelial cell receptor monosialotetrahexosylganglioside (GM_1) (180). Furthermore, should an IgA-coated pathogen contacts an epithelial or adaptive immune cell, it may initiate crosslinking and activate innate and adaptive immune cells. For example, IgA binds to FcαRI (CD89), pIgR, Fcα/μR, transferrin receptor (CD71), Galectin-1 (which is expressed on cells involved in regulation of innate and adaptive immune response in the intestine), and the M cell IgA receptor (199). The IgA attachment to pathogen surface or to secreted pathogen products engage FcαRI present on neutrophils, monocytes, eosinophils, and some macrophages and DCs to induce phagocytosis, promote cytokine release, activation of reactive oxygen species and trigger antibody-dependent cell-mediated cytotoxicity (199). Interestingly, IgA-producing plasma cells influence composition and diversification of intestinal commensal bacteria (200, 186) . An *in vivo* study showed that 24-74% of commensal bacteria in the feces of healthy humans are coated with IgA, significantly more than with IgG or IgM (201). A more recent study in children found that children with intestinal dysbiosis such as coeliac disease have a skewed commensal bacteria population and reduced IgA-coated bacteria compared to healthy individuals (202). The plethora of immune functions of sIgA in mucosal tissues indicates its importance in protection against microbial pathogens and at the same time its key role in maintaining the mucosal immune homeostasis and balance between commensal bacteria and nutrients present in the intestinal lumen.

IgA+ plasma cells secrete inducible nitric oxide synthase (iNOS) and TNFα which are important for IgA production and gut homeostasis at steady state and after challenge with a gut-tropic pathogen (198). The study from Fritz et al showed that B cell-derived iNOS and TNFα play an important function in the generation of IgA^+ plasma cells (203). iNOS is an intracellular enzyme

that works as a catalyst in a reaction which utilizes nutritional L-Arginine to produce nitric oxide and L-citrulline (204). Nitric oxide is an important regulator of cellular metabolism, inflammatory cytokine production, survival and chemotactic signals (198). iNOS activation and nitric oxide production is required for differentiation of Th17 and Treg cells (205, 206) and it controls expansion of activated $CD4^+$ Th1, Th2, Th17 -type T cell pools as well as $CD8^+$ cells in lymph nodes (207, 208, 209). B cells only produce TNFα after they have first been activated by antigen and then activated by CD40L presented on Th2-type T cells (210). It was also reported that TLR1/2 activation of $CD27^+$ memory B cells produces TNFα, GM-CSF, and G-CSF (211) and TLR1/2, TLR7, and TLR9 stimulate CD19+ B cells to produce cytokines IL-1α, IL-1β, IL-6, TNF-α, IL-13, and IL-10, and chemokines MIP-1α, MIP-1β, MCP-1, IP-10, and IL-8 (211). Activated B cell and plasma cell-secreted cytokines exert important immune regulatory roles on the mucosal environment and they are crucial components of the local immune system for protection against pathogens.

1.3.3.2 Adaptive cell-mediated immunity in the intestine

Adaptive cell-mediated immunity is mediated by T cells present in GALT including the intestinal epithelium. T-cells are differentiated by the expressed surface receptors $CD4^+$ and $CD8^+$ cells. CD4+ T cells can be distinguished into distinct subsets by their distinct effector function and cytokine secretion in response to the antigen presented on APCs or cytokines secreted in the intestinal epithelial environment. They play multiple roles in immune protection against intestinal pathogens and they play an important role in maintaining gut homeostasis in an environment rich in food and commensal antigens. Failure of T cells to maintain one of these functions due to genetic

alterations or as an aberrant response to infection can lead to inflammatory disease and damage to epithelial tissue in GALT.

CD4-expressing helper T (T_H) cells and CD8-expressing cytotoxic cells originate from the thymus as precursors without a defined effector function (212). T-lymphocytes have a unique T cell receptors (TCR) on their surface. The pool of TCRs are such that they can recognize an almost unlimited number of antigens but only one TCR is present on each T cell. CD4 helper T cells (T_H) recognize antigens presented in major histocompatibility complexes class II (MHCII) after processing by APCs (212). CD4+ T_H cells further differentiate into effector cells with specific functions in T_H1, T_H2, and T_H17 immunity with cellular plasticity in intestinal tissue (213). CD4+ regulatory Foxp3 expressing T cells (Tregs) are a subset of T cells that play a major factor in immune regulation and tolerance and a high proportion of these subsets reside in intestinal *lamina propria* (178). CD8 expressing cytotoxic T cells recognize antigens presented in MHCI on host cells, deriving from viral or intracellular bacterial pathogens that infected host cells. When $CD8^+$ T cells engage with MHCI presenting intracellular antigen, they are activated and they differentiate into effector cells which results in the production of IFNγ or they have cytotoxic activity directed towards infected cells.

The gut epithelium is home to an abundant population of $CD8^+$ T cells located in between epithelial cells or dispersed in underlying tissue (190). The $CD8^+$ T-cells are distinguished by expression of αβ or γδ TCRs, which determines their specific localization and role in the intestinal immune system (214). The γδ T-cells are intraepithelial lymphocytes (IELs) while αβ T-cells are located in the *lamina propria*. Subsets of γδ T-cells are present in low numbers in the circulation but they are abundant in epithelial tissues and constitute between 10–100% of T-cells in the epidermis of the skin and the epithelial tissues in gastrointestinal tract (215). They play an

important role in maintaining epithelial barrier, regeneration of epithelium, homeostasis and providing a balance between commensal tolerance and pathogen clearance (215).

Human and mice subtypes of γδ T-cells are well characterized and two nomenclature are used today, the German and Heilig & Tonegawa nomenclature (215). The murine γδ T-cell subsets are characterized by Vγ whereas human γδ T-cell subsets are distinguished by Vδ usage (215). A porcine subpopulation of γδ T-cells express CD2 and in combination with CD8α three different subsets can be defined as $CD2^{+}CD8\alpha^{+}$, $CD2^{+}CD8\alpha^{-}$ and $CD2^{-}CD8\alpha^{-}$ (214). The development of γδ T-cells in mice starts in the thymus during the embryonic stage and they migrate to the intestine before birth as influenced by the expression of CCR9 (which is the receptor for CCL25) and α4β7 (which is the integrin which binds MAdCAM-1 receptor) (215). Secretion of cytokines in local tissue has an important effect on IELs maturation and function. Mice deficient in IL-7 or IL-7R in thymic tissue lack γδ T-cells implying the importance of this cytokine and receptor on γδ T-cell generation (216, 217). Also, IL-15 deficient and IL-15R-deficient mice have no Vγ5+ IEL residing in intestinal epithelium which implies that intestinal epithelial cell-derived IL-15 plays an important role in proper localization and tissue tropism of γδ T-cells (218).

The intestinal γδ T-cells are nested between two epithelial cells such that they are able to monitor and react to the signals coming from enterocytes or bacteria from the lumen. They are motile and can move between epithelial cells to actively surveying the environment (215). It has been reported that activated γδ T-cells maintain epithelial homeostasis and tissue repair by secreting keratinocyte growth factor 1 (KGF-1), which induces proliferation of epithelial cells in crypts (219). The importance of γδ T cells in epithelial homeostasis and repair was shown in a study with mice model of chemically induced colitis with dextran sodium sulfate (DSS) (220).

DSS in TCRδ−/− or KGF-1-deficient (KGF1−/−) mice had increased tissue damage and the repair of damaged epithelium was significantly reduced compared to the wild-type (WT) mice (220).

The intraepithelial γδ T-cells play a major role in limiting access of luminal pathogens and commensals to the subepithelial tissue. Due to their intraepithelial and basolateral location, they can sense invading microbes and effectively communicate with epithelial cells and other immune cells to clear the infection. Small intestinal γδ T-cells express cytolytic proteins such as Granzyme A and B, suggesting a potent cytotoxic effect against pathogens and infected cells (221), but other studies reported that porcine γδ T-cells do not express perforin (222). Early *in vitro* experiments found that small intestinal γδ IEL express Th-1 type T-cell markers and upon ligand binding, they destroy target cells by cytolysis (223). A major finding in a study by Ismail et al. 2011was that γδ IEL responded to invasion by resident pathobionts, and exogenous overt pathogens (224). Further, they established that bacterial stimulation of γδ IEL was indirect, and required activation of MyD88 signaling in neighboring epithelial cells (224). *Salmonella* Typhimurium invaded epithelial cells and induced neighboring γδ IEL to express innate antibacterial effectors such as lectin RegIIIγ (224). The activation of γδ IEL provided critical antibacterial protection of the mucosal surface, especially during the first hours following *S.* Typhimurium exposure thus suggesting an important role of γδ IEL at the inception of the immune response (224). This study did not reveal whether antibacterial factors such as RegIIIγ and RegIIIβ were the main protective molecules that directly inhibited bacterial epithelial penetration or whether cytolytic functions of IELs were responsible for the removal of *S.* Typhimurium infected epithelial cells (224).

Activated IELs in the skin and/or gut can generate significant amounts of biologically active soluble mediators, including granzymes, cytokines, and chemotactic factors, thus enhancing the immune response (225). In response to viral infection, IELs respond with the secretion of IFNγ

and cytotoxic activity specifically against virus-infected cells in culture (226, 227). Activated IEL induce the upregulation of IFN-stimulated genes (ISGs) and antiviral response in intestinal epithelial cells *in vitro*, which was linked to the production of type I, II and III IFNs (225). Also, epithelial cells were more impervious to virus infection *in vitro* when supernatants from activated IEL were present (225). When activated T cells induced ISGs in the villus epithelium in mice, it correlated with significantly increased resistance to murine norovirus (MNV) within 40 h of gastrointestinal infection (225). These findings support the notion that IELs are a bridge between innate and adaptive immunity, which facilitate the important transfer of signals from the lumen to the *lamina propria* while maintaining epithelial integrity and protection against pathogens. These cells are conserved from fish to humans and have a limited diversity of receptors thus exhibiting some innate immune traits. Although lacking receptor diversity, γδ T-cells are able to react to stress signals from epithelial cells and conserved PAMPs to induce a protective immune response against extracellular and intracellular pathogens. Further research is needed to elucidate all molecular mechanisms that contribute to the multiple protective functions of γδ T-cells.

In addition to the above-presented roles of intestinal γδ T cells in maintaining gut barrier and protection against different pathogen, one surprising role of IELs emerged from studies of intestinal inflammation. It was reported that IELs are important regulators of inflammation and that in different murine models for IBD, celiac disease and colitis these cells suppressed inflammation and the cytotoxic effect of αβ T-cells (228). In the murine model of intestinal inflammation and colitis, γδ T cells deficient animals had increased levels of IFNγ in the intestinal epithelium and aggravated intestinal inflammation (228). These data confirm previous findings where the transfer of γδ IEL from WT to TCRδ−/− mice resulted in reduced colitis due to increased TGFβ1 production by intestinal IELs, decreased IFNγ expression and protection against

inflammation (229). Findings in a murine model of intestinal inflammation correlate to human patients with celiac disease where a $CD8^+$ TCR $\gamma\delta^+$ $NKG2A^+$ subset of gut γδ T-cells dampened the pro-inflammatory (IFNγ) and cytotoxic (Granzyme B) potential of αβ intestinal T-cells (230). This anti-inflammatory effect of γδ T-cells was partially mediated through the production of TGFβ1 after HLA-E binding to on enterocytes and/or TCR$\alpha\beta^+$ IELs (230). These results further emphasize the important homeostatic role of IELs in intestinal immunity.

Currently, three different effector CD4 populations are described based on the specific cytokine secreted (231). Th1-type T-cells produce IFNγ cytokine, Th2-type T cells produce IL-4, IL-5, and IL-13 (232) and Th-17-type T cell cells produce IL-17. The Th1-type T-cells are involved in the eradication of intracellular pathogens such as intracellular bacteria, viruses, and some protozoa. They are activated by type I interferons (IFNα, IFNβ) and type II interferon (IFNγ) with successive activation of the signal transducer of activated T-cells, STAT-1 and STAT-4 (233). This signal cascade induces T-box transcription factor expressed in T cells (T-bet) and IFNγ (231). IFNγ is a strong activator of innate immune cells such as macrophages and an inducer of class-switch recombination of B cells to immunoglobulin IgG isotypes that opsonize microbes for enhanced uptake by phagocytes (234). Differentiated Th1-type T cells express surface molecules CCR1, CCR5, and CXCR3, which helps them reach sites of inflammation (231).

The Th2-type T-cells secrete cytokines IL-4, IL-5, and IL-13. IL-4 leads to production of IgE antibodies that activate basophils and mast cells for granule release, IL-5 increases eosinophil mobilization and IL-13 increases mucous production and hypermotility (234). The effector Th2-type T cell cytokine secretion facilitates enhanced elimination of parasitic infections, such as helminths from intestinal mucosa. When WT mice were infected with *Schistosoma mansoni* eggs, intestinal DCs were engaged and promoted a Th2-type T cell response (235). NF-κB1 expression

within DC induced production of IL-4, IL-5, and IL-13 in the draining lymph node of WT mice, while NF-κB1$^{-/-}$ mice did not express Th2-type T cell cytokines and developed a polarized antigen-specific IFNγ response (235). The importance of signals from invading parasites on the Th2-type T cell response in humans is shown in the findings that Th2-type T cells are almost absent from intestine from people living in countries with good sanitation with limited exposure to helminths (231).

Th17-type T-cells were discovered as a subset of effector CD4 cells that play an important role in mucosal immune regulation and protection. They play a pivotal factor in many inflammatory and autoimmune diseases that can have devastating effects on mucosal tissues. Th17-type T-cells are characterized by the production of the IL-17 family cytokines, IL-17A and IL-17F (236) which induce neutrophil production by regulating the expression of G-CSF and IL-8 on innate immune cells and epithelial cells (237). Th17-type T-cells also produce IL-22 and GM-CSF (237). IL-22 belongs to the IL-10 cytokine family that facilitate maintenance of epithelial barrier integrity in gut, lung, and skin, and GM-SCF promotes the enhanced production of granulocytes and monocytes in bone marrow (234). Differentiation of Th17-type T cells from naïve CD4^{+} cells is induced by co-stimulation with TGFβ in mice (238) or IL-1β in humans, as well as IL-6 (239), IL-23 and local molecular signals such as nitric oxide (205). Th17-type T-cells are present in great numbers in intestinal tissue and are the dominant adaptive immune cells that respond to extracellular bacteria and fungi (240). Interestingly, germ-free mice do not have CD4+ Th17-type T-cells in the intestine and IL-7 and IL-22 secreting Th17-type T cells are induced with the introduction of SFB in the intestine (241). SFB-colonized mice had increased expression of genes associated with inflammation and anti-microbial defenses which resulted in improved resistance to the murine intestinal pathogen *C. rodentium* (241). A recent study revealed that a

central role in regulating the relationship between SFB and Th17-type T cell is facilitated by IL-17RA, a receptor on epithelial cells (242). Mice deficient for IL-17RA had increased SFB numbers due to decreased expression of α-defensins, *Pigr,* and *Nox1* (242). Also, IL-17RA-deficient mice had a significantly lower concentration of sIgA in the intestinal lumen which may suggest that IL-17RA is required for expression of pIgR and IgA secretion to intestinal lumen (242). The regulation of pIgR by Th17-type T-cells was also reported in association with mucosal immune protection against intestinal parasite Giardia (243). Furthermore, when experimental autoimmune encephalomyelitis was induced in IL-17R signaling-deficient mice, they exhibited increased intestinal *Csf2* expression and elevated systemic GM-CSF cytokine concentrations, which resulted in exacerbation of autoimmune disease (242). Experimental data in mice regarding immune cross-talk between SFB and Th17-type T-cells and immune regulation cannot be easily extrapolated to humans due to the relative absence of SFB in the human intestine. It seems rational to speculate that in human intestine, other species of commensal bacteria have the same regulatory role as SFB in mice due to the high prevalence of Th17-type T-cells in the human intestine. Indeed, studies where human fecal matter was transplanted into germ-free mice, revealed that human commensal bacteria, *E. coli,* and *Bifidobacterium,* were able to induce a Th17-type T response in mice (244, 245). Induction of Th17-type T-cells was initiated by close adhesion of commensal bacteria to the surface of epithelial cells (245). This was also supported by the findings that majority Th17-type T-cells, but not other T-cells, identify specific antigens expressed by *SFB* in mice (209). This recognition is dependent on MHCII expression of the antigen by $CD11C^+$ (246) and monocyte-derived macrophages (247). Upon activation of specific $ROR\gamma t^+$ $CD4^+$ T cells, they receive a signal from epithelial cells reacting to SFB adhesion. Attached SFB induce epithelial cells to produce serum amyloid A (SAA) and ROS which promote IL-17 production and Th17-type T-cells homing

to the small intestine (245, 248). This epithelial response is further enhanced by IL-23-dependent IL-22 production by the ILC3, and IL-1β production from *lamina propria* $CD11C^+$ which altogether results in amplification of local IL-17 production by Th17-type T cells (245, 248). This complex mechanism of Th17-type T-cell immune response activation, mutual dependence of commensal bacteria and multiple cells and molecular signals in intestinal tissue presents a challenge regarding intestinal inflammatory diseases. It appears that the Th17-type T-cell pathway is central in the underlying mechanism behind immune-mediated intestinal inflammatory diseases.

If not controlled by regulatory immune mechanism, the activation of effector subtypes of CD4+ T-cells in intestine would lead to continuous inflammation and destruction of intestinal tissue. The important regulators of innate and adaptive immune cells and inflammation are T regs. Tregs cells are characterized by expression of CD4, CD25, FoxP3 and they constitute 10-20% of all CD4+ cells and 20-40% of all CD4+ cells in colon (178, 249). The proportion of FoxP3+ T cells compared to effector CD4+ cells is higher in the intestine than in other tissues, which suggest their importance in regulating immunity and inflammation in intestinal *lamina propria* and mucosa (178). Recent studies revealed the existence of two subsets of $FoxP3^+$ CD4+ T cells in mice, thymically-derived natural (n)Treg cells that co-express neuropilin 1 (NRP1) and Helios and peripherally induced (p)Treg cells (or iTreg) which are $NRP1^-$ $Helios^-$ $Foxp3^+$ (178, 250). These two Treg -type T cell subtypes differ not only in marker expression and induced localization but they also have some unique characteristics. The nTreg cells are induced in thymus with the engagement of TCR and presence of TGFβ and IL-2 with self-antigen specificity and cell contact-dependent mechanism of suppression (251). The pTregs are induced naïve T-cells in secondary lymphoid organs or inflamed tissue which acquires FoxP3 expression with co-stimulation of CTLA-4 and cytokines TGFβ, IL-2 and retinoic acid which are abundant in intestinal tissue (252,

251). They recognize self and foreign antigens and facilitate immune suppression with secreted cytokines (251). These pTregs are further distinguished by the expression of RORγt on $FoxP3^+$ $ROR\gamma t^+$, which are more prevalent in the colon, and $FoxP3^+$ $ROR\gamma t^-$ which are dominant $FoxP3^+$ T cell subset in the small intestine (250, 253). Their affinity for a specific region of the intestine may represent their specific function in the regulation of the intestinal immune system. $FoxP3^+ROR\gamma t^+$ pTregs are closely connected to the presence of microbiota in colon and germ-free mice or antibiotic-treated mice have reduced numbers of $FoxP3^+$ $ROR\gamma t^+$ pTregs (194, 250, 253). $FoxP3^+$ $ROR\gamma t^-$ cells are more prevalent in the small intestine and mice fed with antigen free food have significantly decreased numbers of these cells in the small intestine, which suggest that antigens promote clonal expansion of these cells (194). Also, deficiencies of $FoxP3^+ROR\gamma t^+$ pTregs result in increased intestinal Th2 (254), Th1, and Th17 type T cell responses (250), while deficiencies of $FoxP3^+ROR\gamma t^-$ cells lead to greater immune reaction towards dietary antigens (194).

The pTreg cells maintain the immune balance in intestinal tissue by suppressing immune responses and maintaining self-tolerance. Multiple mechanisms of pTreg suppression were reported from which the production of cytokines IL-10 and TGFβ, and direct contact suppression of DC were supported by most studies. IL-10 is responsible for inhibited expansion and function of conventional T cells, inhibition of activated macrophages and DCs, and inhibition of pathogenic Th17-type T cell responses (255, 256, 257). It specifically binds to IL-10R1 on many innate and immune cells (258). The importance of Treg derived IL-10 was examined in the study by Robtsov et al 2008. They showed that particular removal of IL-10R expression in Treg cells led to spontaneous colitis suggesting that Treg-derived IL-10 plays an important role in preventing inflammation at mucosal surfaces (157).

TGFβ is a regulatory cytokine that plays an important role in essential physiological processes such as embryogenesis, cell differentiation and immune response (259). The TGFβ family consists of three members, TGFβ1, TGFβ2, TGFβ3 with TGFβ1 being prevalent type in the immune system of mammals (260). It is produced in multiple cells as a latent form that needs to be proteolytically cleaved to become active after which it binds to TGF-type I and TGF-type II receptors (259). Binding to the receptors initiates phosphorylation of downstream targets and activates multiple signaling pathways (259). The central role in this signaling pathway is provided by action of SMAD transcription factor (the abbreviation refers to the homologies to the Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") family of genes), which binds to multiple transcription factors and results in diverse cellular responses (261). TGFβ is produced by a variety of innate and immune cells and has diverse roles in the regulation of immune response. Although TGFβ is produced by pTreg cells, it also has an important function in the transformation of naïve T cells in peripheral tissues into FoxP3+ pTreg cells (252). The effect of TGFβ on pTreg differentiation is further aided by T cell-secreted IL-2 (262). The induction of pTreg cells is particularly evident at intestinal mucosal sites where $CD103^+$ GALT DCs play a major role in inducing that mechanism (259). The production of TGF-β and RA from $CD103^+$ cells are prerequisite factors for conversion of naïve T cells to pTreg cells in the periphery (263). Other external signals, such as specific bacterial species or their products, can initiate pTreg cell differentiation in the intestinal mucosa. *Clostridia* species induce mucosal TGFβ and $FoxP3^+ROR\gamma t^+$ pTregs formation (264) and short chain fatty acids (SCFA), butyrate, and propionate, act directly on $CD4^+$ cells or indirectly via DCs to secrete TGFβ and promote $FoxP3^+$ generation in intestinal tissue (178). TGFβ secreted by the intestinal *lamina propria* pTreg cells is a major regulator of effector T-cell differentiation and effector

functions. It has a strong inhibitory effect on Th1-type and Th2-type T cell differentiation *in vitro* via downregulation of T-bet and GATA-3 expression (259). It also regulates differentiation of Th17-type T cells but it has a dual effect depending on cooperation with other cytokines. TGFβ alone promotes FoxP3 expression and pTreg cell differentiation from activated $CD4^+$ cells but the addition of IL-6 and TGFβ together in T cell *in vitro* culture leads to differentiation of effector Th17 cells (265). These findings reveal how cytokines can have multiple and diverse roles in immune response depending on tissue-specific signals and external factors. Additionally, TGFβ plays an important role in CSR such that mice deficient for the TGFβ receptor TβRII have diminished IgA production (191). IgA isotype switching is induced in response to TGFβ in concert with other cytokines such as IL-2, IL-4, IL-5, IL-6, and IL-10 from different intestinal cell populations (132). This response again highlights how the effect of one cytokine is influenced in the context of multi-cytokine environment.

As well as mediating suppression through cytokine secretion, pTreg cells also exert suppression of NK cells, DCs, macrophages, monocytes, neutrophils, T and B cells by direct contact (266). pTreg cells express CTLA4 and TIGIT (T cell immunoreceptor with Ig and ITIM domains) which directly act on DCs to inhibit expression of CD80 and CD86 which leads to IL-10 secretion and suppression of effector T cells (267, 268). In return, DC antigen presentation and the local abundance of TGFβ, IL-2, and retinoic acid in intestinal *lamina propria* favours pTreg cell generation (251). There is a feedback regulatory loop between DCs and T reg wherein loss of DCs leads to a loss of Treg cells and/or the remaining Treg cells exhibit decreased Foxp3 expression (269). DC-dependent depletion of Treg cells leads to increased number of effector T cells and the production of inflammatory cytokines, such as IFNγ and IL-17 (269). Furthermore, increasing number of DCs leads to increased number of Treg by a mechanism that requires MHCII

expression on DCs (269). This mutual dependence and regulation of Treg cells and DCs is a dominant mechanism in gut mucosal tissue. *In vitro* assays showed that *lamina propria* and MLN CD103+ DCs are able to promote the generation of $CD4^+FoxP3^+$ cells from naïve $CD4^+$ cells by production of retinoic acid (263). On the contrary, $CD103^-$ DCs can convert naïve $CD4^+$ cells to effector cells and induce secretion of pro-inflammatory cytokines (263). A study from Manoharan et al 2014 showed that DCs cells ability to regulate induction of Treg cells relies on TLR2 signaling via AKT activation of β-catenin/T cell factor 4 which induces expression of IL-10 and retinaldehyde dehydrogenase 2, and suppresses expression of pro-inflammatory cytokines (270). Knock out of β-catenin gene from DCs induces Th17/Th1-type T cell generation in response to zymosan (270). This activation mechanism of β-catenin in DCs via TLR2 stimulation is suggested to be a mechanism of controling autoimmune inflammation (270).

Additional mechanisms of Treg-induced suppression of effector cells and in regulation of inflammation have been reported. Treg cells express CD39 and CD73 (two ATP diphosphohydrolases) which degrade ATP to adenosine via an AMP intermediate (271). Also, Treg cells bind IL-2 in higher capacity and therefore can out-compete effector cells for this essential T cell activation cytokine (272). Discovery of the novel Ebi3–IL-12a heterodimeric cytokine, interleukin-35 (IL-35), revealed an additional tool for Treg dependent immune suppression as ectopic expression of IL-35 can regulate activity on naive T cells and suppresses T-cell proliferation (273).

Although immune suppression and self-tolerance is an essential process during steady state and tissue repair, increased activation of pTreg was reported in cases of chronic viral, bacterial and parasitic infections. It was found that *Helicobacter pylori* infection-induced activation of antigen-specific $CD4^+CD25^+$ regulatory T-cells, which suppressed memory T-cells in the gastric and

duodenal mucosa (274). Higher numbers of CD4+FOXP3+T cells were found in areas of duodenal gastric metaplasia in the ulcer patients compared to duodenal gastric metaplasia of asymptomatic individuals and in healthy mucosa (275). Interestingly, pathogen-specific Treg cells were induced in response to chronic *Listeria monocytogenes* infection of mice but authors reported that *L. monocytogenes* infection selectively primed for proliferation, expansion and subsequent contraction of *L. monocytogenes* specific $Foxp3^-$ effector $CD4^+$ cells (276). Additionally, this study found that the numbers of *L. monocytogenes*-specific $Foxp3^+CD4^+$ regulatory cells stayed unaltered (276). The authors concluded that selective priming and expansion of $Foxp3^-$ CD4 T cells is a distinctive characteristic for acute bacterial infection (276). Taken together, these studies indicate that there was pathogen-specific induction of a distinct subset of Treg cells which favours pathogen persistence.

Each effector $CD4^+$ subset is activated by antigen presentation by APCs. The local tissue-specific cytokines present during APCs internalization of antigen leads to changes in the APC's molecular signals, which manifest as secretions of specific cytokines that in turn influence CD4+ T cell differentiation. APC-derived cytokines induce transcriptional cascade that regulates specific effector T cell differentiation with their own characteristic cytokine signature profile. These $CD4^+$ T cell-derived cytokines maintain the specific lineage self-preservation through positive feedback, and they regulate suppression of other lineages (231). Furthermore, effector CD4 T cell-derived cytokines enhance the activation and effect of specific innate immune cells responsible for the eradication of specific antigen. For example, Th1-type cell-derived cytokines induce monocytes/macrophage phagocytosis and inflammation to destroy intracellular pathogens or cancer cells; Th2-type cell-derived cytokines attract and activate eosinophils, basophils and mast cells to fight off parasites, and Th17-type T cells recruit neutrophils and enhance their killing effect

of extracellular bacteria and fungi (234). Although this is a widely accepted division of $CD4^+$ lineages, recent research into their development, phenotype and functions revealed that $CD4^+$ T helper cells may have plasticity which results in some phenotypically and functionally intermediate subtypes. It was reported that *Candida albicans*-specific Th17-type T cells produced IL-17 and IFNγ, but not IL-10, whereas *Staphylococcus aureus*-specific Th17-type T cells produced IL-17 and were able to produce IL-10 after re-stimulation (277). Cytokines IL-6, IL-23, and IL-1β produced by activated APCs promote Th17-type T cell differentiation as a response to both pathogens, but IL-1β secretion was crucial in *C. albicans*-induced Th17-type T cell differentiation to oppose the inhibitory activity of IL-12 and to prime IL-17/IFNγ double-producing cells (277). Further, this study showed that IL-1β inhibited IL-10 production in differentiating and in memory Th17-type T-cells, whereas blockade of IL-1β *in vivo* led to increased IL-10 production by memory Th17-type T cells (277). This Th1/Th17-type T-cell plasticity plays an important role in the inception of inflammatory diseases in the intestine. The implication of intermediate Th lineage in colitis was studied using a Th17-type T-cell transfer model of colitis (278). This study found that IFNγ–deficient Th17-type T cells retained an $IL\text{-}17A^+$ phenotype and were unable to induce colitis in recipients and that development of disease required the transition of a subset of Th17-type T-cells precursors to Th1-like T-cells (278). Both STAT4 and T-bet were highly expressed, but not the IL-12 or IFNγ receptors. It was suggested that Th17-type T-cells may induce signals for the development of pathogenic Th1-type T-cells from naïve precursors (278). From these result, authors concluded that Th17-type T-cells are important factors responsible for colitis pathogenesis acting in two ways: by directly transitioning to Th1-like T-cells and by supporting the development of classic Th1-type T-cells (278).

As well as the above-explained role in inflammation and pathology, change of phenotype and cytokine secretion of Th17-Type T-cells has also been reported during immune homeostasis and suppression of inflammation. In a study using two new fate-mapping mouse models, authors showed that CD4 T-cells that formerly expressed IL-17A had the ability to change phenotype and become anti-inflammatory CD4 Treg cells (279). This change was depicted in a complete shift of transcriptional profile from Th17-type T-cells into regulatory T-cells with the important role of canonical TGFβ signaling and consequently of the aryl hydrocarbon receptor (AhR) in this conversion (279). This study showed an important mechanism of Th17-type T cells trans-differentiation into regulatory cells, which led to the resolution of inflammation and restoration of immune homeostasis (279).

1.3.4 Immune response to infection with *L. intracellularis*

(Modified version of this chapter was used to write a review article "Immune response and protection against *Lawsonia intracellularis* infections in pigs", Obradovic M. and Wilson L. H.; submitting to Veterinary Immunology and Immunopathology)

L. intracellularis has two clinically distinct forms of the disease, PIA and PHE, which differ in severity, clinical symptoms, and ages of animals impacted by them. *L. intracellularis* infection induces distinct immune response with unique cellular and molecular immune patterns in the intestine, which are not fully elucidated. Generally, *L. intracellularis* infection leading to PIA promotes induction of a mild immune response with limited infiltration of B cells, $CD8^+$ and $CD25^+$ T lymphocytes in the *lamina propria* of jejunum, ileum, and colon (Fig. 1.6A, C) (13, 3). In pigs that have the hemorrhagic PHE form of the disease, the cellular response is greater but still

show moderate infiltration of $CD8^+$ and $CD25^+$ T lymphocytes into *lamina propria* (Fig.1.6B, D) (13). IgA-bound *L. intracellularis* within intestinal epithelial cells are observed in both PIA and PHE (Fig.1.6) (13). Because *L. intracellularis* is the sole species responsible for both disease models without obvious differences in pathogenicity between isolated strains, it is reasonable to speculate that different clinical symptoms are due to factors related to the host animal that could range from genetic base of immunity of different breeds, age of animals, maturity of immune system at time of infection, presence of specific maternal antibodies, parity of sows, presence of specific commensal bacteria, nutrition, etc. Changes in immune response and gene expression in response to infection have been studied to decipher the complicated host-pathogen immune interactions, which impacts *L. intracellularis* pathogenesis.

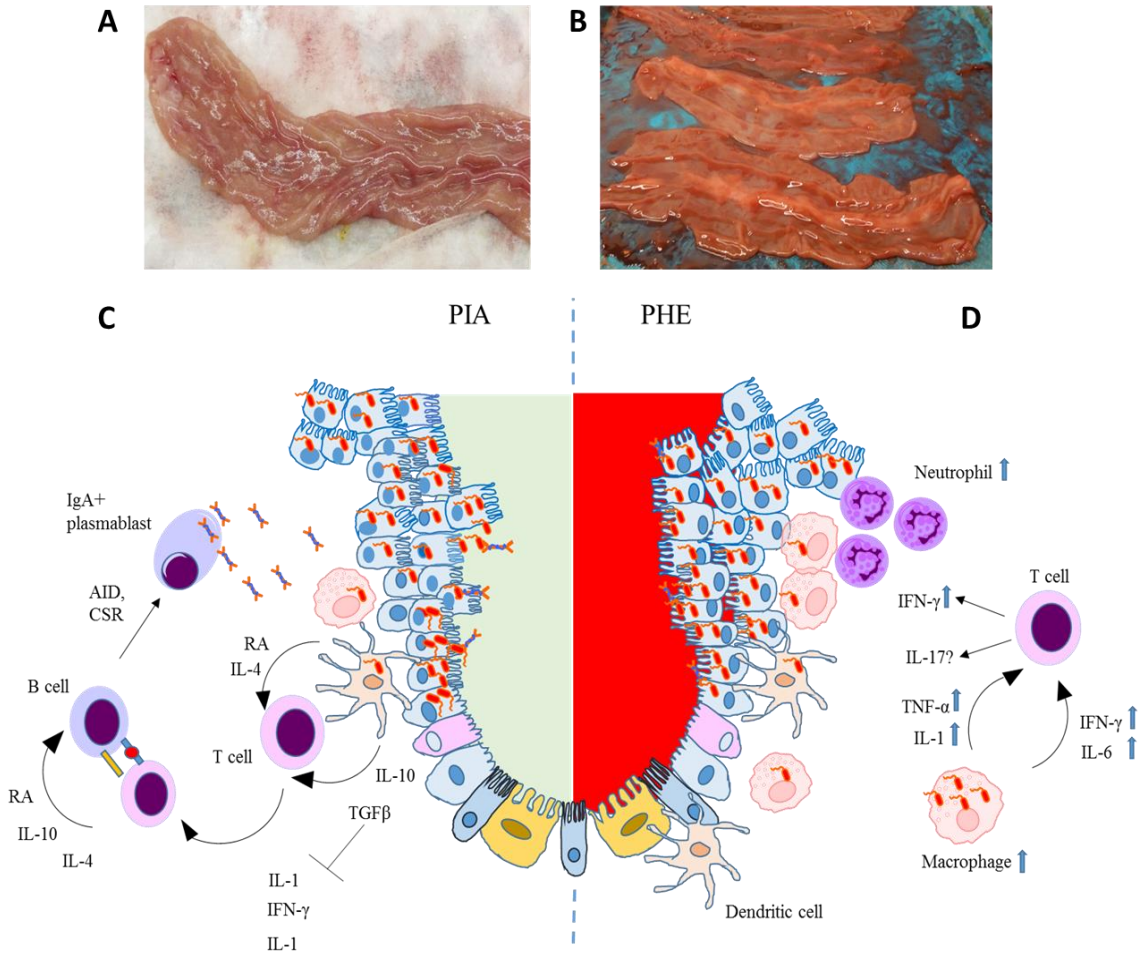


Figure 1.6. Early mucosal immune response to *L. intracellularis* infection in PIA and PHE form of disease. Ileum of *L. intracellularis* experimentally infected piglet, PIA form of disease (A) and ileum from gilt with a field case of PHE from Saskatchewan (B). In PIA form of ileitis, microscopic changes appear as multiplication of immature cells (light blue cells), absence of goblet cells and disturbance of normal crypt cell differentiation (C). Mucosal IgA titers and cytokine production against *L. intracellularis* are delayed which may aid to bacterial spread into the *lamina propria.* At 5 days post-infection (dpi), bacteria were detected in *lamina propria* surrounding heavily infected crypts. After 11 dpi, significant numbers of *L. intracellularis* were detected in the cytoplasm of hyperplastic crypt epithelial cells and in the cytoplasm of mononuclear cells in the *lamina propria.* Innate and adaptive immune responses were delayed or very mild, possibly due to the suppression of immune response by bacteria. In PHE form of disease (D), inflammation and mucosal hemorrhages is major hallmark of this acute form of ileitis disease. Infiltration of macrophages and neutrophils with secretion of inflammatory cytokines into *lamina propria* lead to the characteristic symptoms of PHE and subsequent death of animal. (Keys for cells on Fig 1.3)

The precise molecular mechanisms in the bacteria and the contributing environmental conditions needed for *L. intracellularis* invasion are still obscure. However, it is known that *L. intracellularis* can infect small intestinal epithelial cells in as few as 12 h post-infection, and it can form discrete foci of bacteria 36 h post-infection (158). At 5 dpi, bacteria were detected in *lamina propria* surrounding heavily infected crypts (Fig. 1.6C) (158). As the infection progresses, bacteria multiply inside crypts and by 8 dpi, multiple bacteria were detected in ileal crypt enterocytes and in the tip of villi without obvious signs of hyperproliferation of the epithelial cells or inflammation (7). In a study from MacIntyre et al 2003, two out of four infected 7 weeks old pigs showed crypt epithelial hyperplasia with evidence of neutrophil infiltration between crypt epithelial cells 7 dpi but without apparent inflammatory reaction in *lamina propria* (13). After 11 dpi, significant numbers of *L. intracellularis* were detected in the cytoplasm of hyperplastic crypts and in the cytoplasm of mononuclear cells in the *lamina propria* (Fig. 1.6C) (7). Interestingly, although bacteria had been detected in mononuclear cells of the *lamina propria* since day 5, no evidence of inflammation was obvious 11 dpi, indicating that innate and adaptive immune responses were delayed or very mild (158, 7). These studies indicate that in the acute phase of infection, *L. intracellularis* thrive in the small intestinal lumen, survive commensal competition, traverse the mucus barrier to infect their host cells and avoid the immediate effect of innate immune cells and cytokines during initial infection.

L. intracellularis infection can lead to changes in serum cytokine expression over time. For instance, 5 week-old *L. intracellularis* seronegative pigs orally infected with 5.25×10^8 *L. intracellularis* showed increased serum TNF-α, IFNγ, and IL-6 after 5 dpi which peaked on 10 dpi for TNF-α and 20 dpi for IFNγ which remained elevated until 40 dpi, and which peaked on 20 dpi and 40 dpi for IL-6 peaked (280). *In vivo* infection studies were performed to gather data on

expression changes in genes that contribute to cell-mediated immunity (281). After 21 dpi, pigs enterocytes showed down-regulation of the gene coding for signaling lymphocytic activation molecule (SLAM7) (also known as CD2), which is required for T-cell activation (78). This study also reported the significant up-regulation of genes encoding MHC-I in infected enterocytes, which implies that the molecular mechanism of presentation of *L. intracellularis* antigens on the basolateral membrane of enterocytes was unaltered, and that the cells could present antigens for induction of adaptive cell-mediated immunity (78). Microarray and cytokine analysis of field cases of pigs with diarrhea showed limited gene expression and limited cytokine secretion in both sera and intestine which points to the conclusion that immune response was weakly induced (281). Interpretation of this study should be undertaken with caution due to the high prevalence of porcine circovirus 2 in intestinal samples which could skew the cytokine expression and interpretation of data.

The root cause of the delayed immune response to *L. intracellularis* and modest infiltration of immune cells at the beginning of infection may lie in the unique way that *L. intracellularis* infects immature crypt epithelial cells. As explained in detail in Section 1.2.4, *L. intracellularis* promote cellular proliferation by altering the β-catenin/Wnt and Notch signaling pathways (84). Abundant β-catenin staining was detected in the cytosol and cytoplasmic membrane of crypts at 7 and 14 days post infection compared to uninfected or resolving crypts (84). β-catenin activity in intestinal DCs and macrophages is an important regulator of intestinal homeostasis (282). TLR2-activated DCs may induce activation of the β-catenin/T cell factor 4 (TCF4) pathway which may lead to DCs production of retinoic acid and IL-10 thus inducing a regulatory state in the intestine and inhibiting Th1-type and Th17-type T cell responses (270). In the absence of β-catenin, activated Th1- and Th17-type T-cell responses induced uncontrolled inflammation and

autoimmunity as a response to zymosan (270). Elevated levels of β-catenin in crypt epithelial cell cytoplasm during *L. intracellularis* infection was not investigated in terms of DCs activation and immune response, but increased serum IL-10 from 5 to 10 dpi and TGFβ from 0 to 5 dpi was reported in a recent study of systemic cytokine response in pigs infected orally with a South Korean strain (280). Although this study examined serum cytokine levels that might not faithfully represent local immune response in the intestinal mucosa, a study from Nogueira et al 2013 reported that a 10-times oral dose of Enterisol® (a commercially available inactivated *L. intracellulairs* vaccine) induced significantly higher levels of TGFβ and TNFα in mucosal scrapings compared to pigs administered the vaccine i.m. (283). This 10-times oral dose of avirulent vaccine completely protected animals from the avirulent challenge thus these cytokine levels may represent an optimal balance between inflammatory and immune suppressive cytokines needed to protect against *L. intracellularis* (283). Serum cytokine levels should be further investigated as possible correlates of protection for future vaccine development.

Although it has not been investigated directly, one molecular mechanism by which *L. intracellularis* may initiate proliferation may be due to the β-catenin pathway-dependent activation of DCs which could lead to induction of an immune suppressive state in the first days of bacterial infection. This delayed or suppressed immune response may help the bacteria to survive, multiply and spread inside intestinal crypts. Further studies are needed to elucidate the relationship between β-catenin expression, *lamina propria* DC activation and immune regulation during *L. intracellularis* infection of enterocytes.

L. intracellularis infection eventually leads to characteristic macroscopic and microscopic pathological changes and alterations of immune cell infiltration from 14 to 24 dpi (Fig. 1.7) (7, 13, 283, 84). By 15 dpi, bacteria spread through small and large intestine with signs of diffused

immature epithelial cell hyperplasia in the ileum, cecum, proximal and spiral colon, and jejunum (7). During this time, bacterial antigens can be detected in crypt cells and the *lamina propria* in the ileum and jejunum, in the lumen and cytoplasm of crypt cells in the duodenum, proximal and spiral colon, cecum and rectum (7), as well as in the lymphoid nodules of submucosa of the proximal colon (7). These findings indicate that *L. intracellularis* were able to spread through the intestinal mucosa unrestrained from immune system and by 15 dpi, bacteria had been taken up by antigen presenting cells and macrophages in *lamina propria* (Fig. 1.7). Here they were presented to adaptive immune cells in lymphoid tissue, which initiated a specific adaptive immune response. These findings confirm previous studies where a significant increase of macrophages with bacteria antigen was detected in intraepithelial cells adjacent to heavily infected crypts at 14 dpi (Fig. 1.7) (13, 284). The involvement and activation of macrophages may explain the sudden increase in pro-inflammatory cytokine around this time point reported in the study of systemic cytokine response in pigs infected with *L. intracellularis* (280). TNFα was the first cytokine to be elevated in response to *L. intracellularis* infection with an increase from 5 to 10 dpi, followed by elevated IFNγ and IL-6 secretion from 10 to 20 dpi (280). These pro-inflammatory cytokines play an important role in activation of adaptive immune cells and specific protection against intestinal pathogens but, in a case of *L. intracellularis* infection, the cytokines could also provide a pro-inflammatory environment contributing to uncontrolled inflammation in intestinal mucosa and PHE form of the disease.

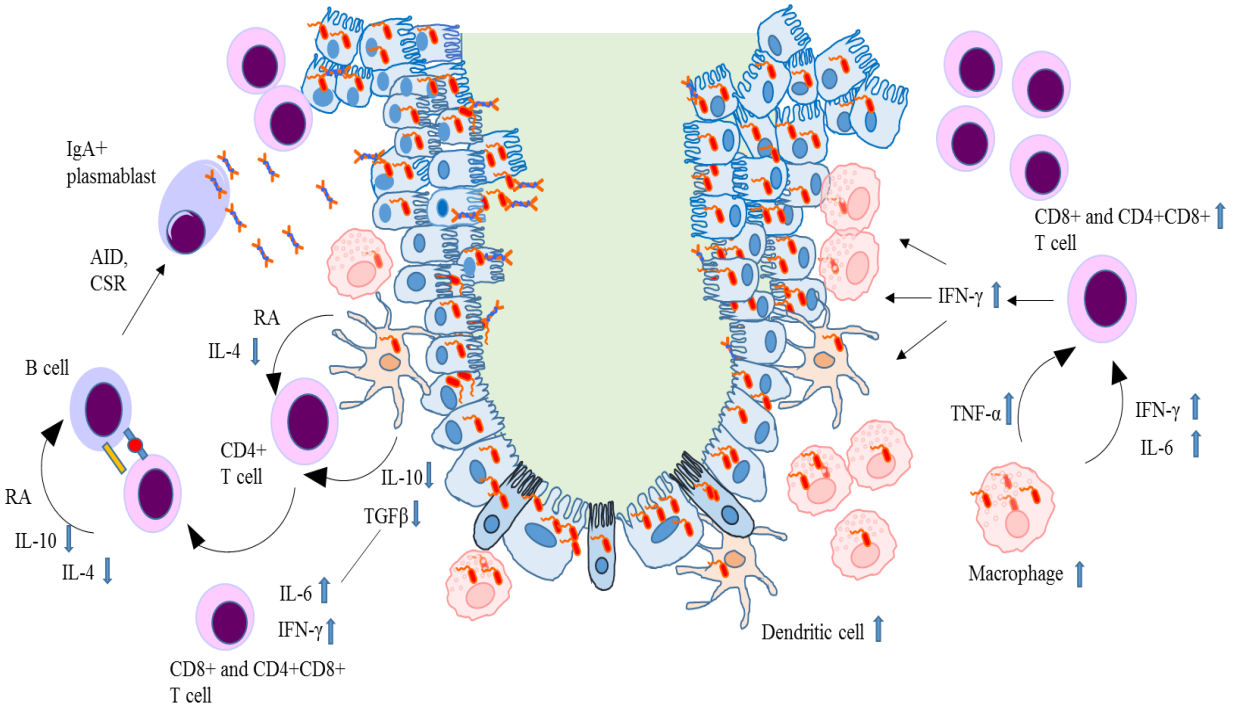


Figure 1.7. Mucosal immune response to *L. intracellularis* infection between 14 and 25 dpi in PIA form. The peak of infection corresponds to microscopic changes characterized by excessive proliferation of immature cells (light blue cells), absence of goblet cells (green cells in Fig. 1 and 2) and increased of numbers of macrophages (pink cells) and dendritic cells with ingested bacteria. The cytokine profile is dominated by increased secretion of IFNγ, TNFα and IL-6 while serum concentrations of IL-10, IL-4 and TGFβ are reduced. The local humoral immune response is activated and there is increase of secretion of specific IgA despite IL-10, IL-4 and TGFβ being reduced. The main source of IFNγ are CD8+ and CD4+CD8+ T cells with minor secretion from CD4-CD8- T cells. Towards the end of this period of peak of the infection, the regulatory arm of immune response is initiated, characterized by secretion of TGFβ at 20 dpi and IL-8 and IL-10 at 25 dpi in serum.

From 19-24 dpi, increased evidence of histopathology were evident, especially in the jejunum where some studies indicate mucosal necrosis (7). Interestingly, IFNγ and IL-6 cytokines in serum concentrations were increasing during the same period while anti-inflammatory cytokines IL-4 and TGFβ were decreased until day 20 or until day 25 for IL-10 (280). Furthermore, independent studies reported increasing IgA titres in intestinal lavages from 15 dpi, ranging from 1:4 to 1:16 (7) and 1:4 to 1:64 (285). These data indicate that the adaptive immune system had

been activated to clear bacteria from the infected intestinal mucosa. Activation of the adaptive immune system is also obvious in ileocecal lymph nodes which showed prominent germinal centre and medullary cords filled with plasma cells from 5 to 24 dpi and increased IgA titres in mucosal scrapings between 15 to 29 dpi (7). Elevated IgA was present despite histological analysis showing reduced B cells numbers in *lamina propria* during the same period (13, 285, 7). Indeed, by 29 dpi most of the bacteria were cleared from intestinal epithelial cells and bacterial antigen was detected mostly in the cytoplasm of mononuclear cells in the *lamina propria* (7). From 35 to 42 dpi, no histological lesions or *L. intracellularis* antigen were detected in intestinal sections indicating clearance of bacterial infection and resolution of intestinal homeostasis (Fig. 1.8) (13, 7). Evidence of clearance of infection was also represented in serum cytokine concentrations. IFNγ levels had increased sharply from 10 to 20 dpi and stayed elevated from 20 to 35 dpi with a small increase on day 40 (280). At the site of infection, there was increased numbers of IFNγ producing cells detected by ELISpot from 9 to 91 dpi (48). IFNγ is an important cytokine for protection against intracellular pathogens and it may be a crucial factor that mediates protection against *L. intracellularis* infection. The significance of IFNγ for mediating protection against *L. intracellularis* was observed when IFNγ receptor knock-out mice failed to resolve infection after 35 days whereas WT mice cleared infection after 21 days (286). IFNγ producing cells were $CD8^+$ and $CD4^+CD8^+$ lymphocytes with a small percentage being $CD4^-CD8^-$ (Fig. 1.8) (284). $CD8^+$ and $CD4^+CD8^+$ lymphocytes have been reported as the main mediators of immune protection (287) but whether cytotoxic T cells, NK cells, or γδT cells are the main source of IFNγ is unclear.

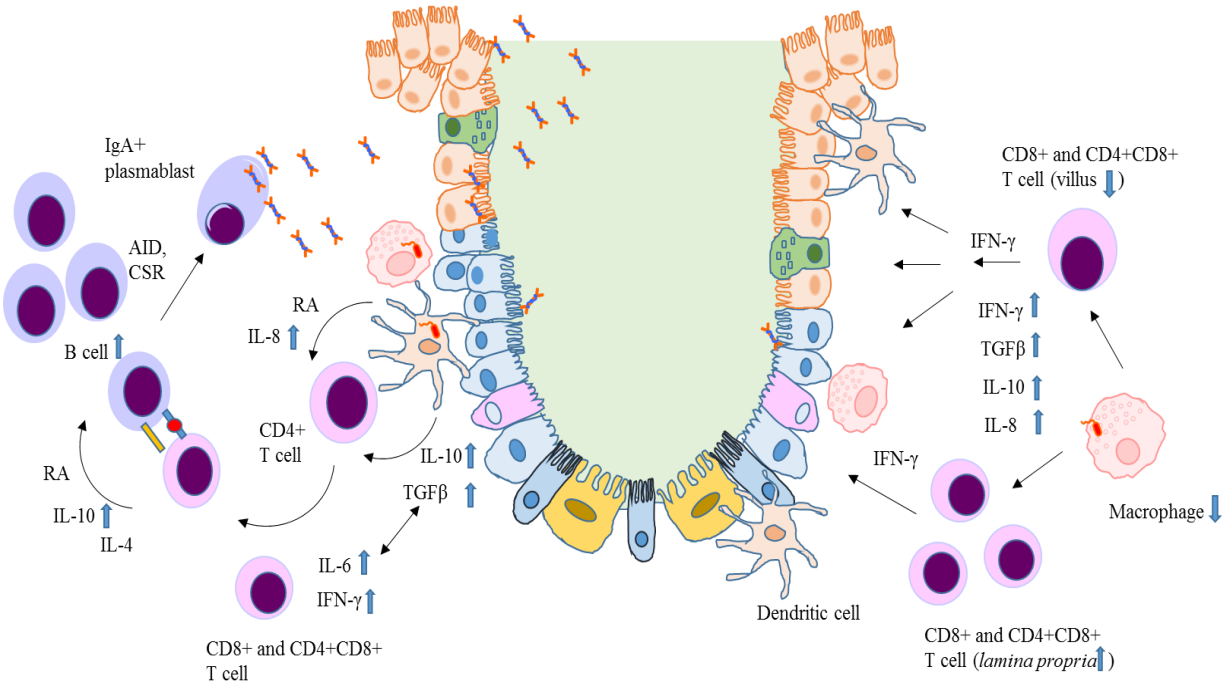


Figure 1.8. Mucosal immune response at the resolution of *L. intracellularis* infection between 26 and 42 dpi in PIA form. Resolution of infection is characterized by absence of histological lesions, clearing of bacteria and restoration of normal crypt differentiated cell population**.** Serum IFNγ levels are increased from 10 to 20 dpi and stayed elevated from 20 to 35 dpi with a small increase on day 40. Increased numbers of IFNγ producing cells are detected in the lamina *propria,* from 9 to 91 dpi. Serum concentration of TGFβ, IL-8 and IL-10 are increased. Relative to 14 dpi, total numbers of CD4+ and CD8+ cells are not statistically reduced at 42 dpi but there is evidence that there are significantly reduced numbers of CD8+ cells in the villous relative to the *lamina propria.* At resolution of infection at 42 dpi, there is a significant reduction in *lamina proprial* CD3+ cells. Successful resolution of infection and subsequent immune protection may depend on the balance between pro-inflammatory and regulatory cytokines, IFNγ, TGFβ, IL-10 and antigen-specific IgA titres and immune cells that are responsible for their secretion.

At resolution of infection (approximately 42 dpi), there is a significant reduction in *lamina propria* of $CD3^+$ cells (13). Relative to 14 dpi, total numbers of $CD4^+$ and $CD8^+$ cells were reduced slightly with no statistical significance compared to 42 dpi but significantly reduced numbers of $CD8^+$ cells were evident in the villous relative to the *lamina propria* (Fig. 1.8)(13). Also, a significant reduction of B-cells in *lamina propria* was detected on day 14 compared to resolved

lesions on day 42 pi (13). MHCII staining revealed a reduction in overall amount of MHCII positive staining concentrated in the core of the villi and in the *lamina propria* after 42 dpi indicating reduced APCs in the *lamina propria* during resolution (13). These data indicate that the adaptive immune response was delayed up to at least 14 dpi, which may help bacterial multiplication and spread throughout the intestine.

During the period of clearance of bacteria and resolution of gut homeostasis, pigs showed an increase in serum anti-inflammatory cytokines such TGFβ after 20 dpi and IL-10 and IL-8 25 dpi (280). These cytokines play an important role in regulating immune homeostasis by limiting effect of pro-inflammatory cytokines and maintaining the intestinal epithelial barrier. Following the course of *L. intracellularis* infection through analyzing the cell population involved, mucosal IgA titres and cytokine production against *L. intracellularis* was delayed during the first 5 dpi which helps bacteria to spread and move into the *lamina propria.* Eventually, the adaptive immune system was primed by day 15 and both cellular and mucosal humoral immunity, represented by IFNγ secretion cells and elevated IgA response, respectively, was activated which help clear bacteria by day 35. It appears that successful resolution of *L. intracellularis* infection requires adequate and timely activation of regulatory arm of the immune system, involving TGFβ and IL-10. These cytokines suppress excessive inflammation, downregulate pro-inflammatory cytokines and cells and help in the recovery of epithelial intestinal integrity. If the inflammatory response was augmented due to increasing pro-inflammatory cytokine secretion or lack of regulatory cytokines or cells, *L. intracellularis* infection led to development of the severe, hemorrhagic form of PE, which often leads to acute clinical symptoms and high mortality rates.

Numerous unresolved questions concerning immune response during the course of *L. intracellularis* infection remain. Although the involvement of serum and mucosal cytokines during

infection has been reported, the involvement of Th17-type T-cell immune response is still unknown. The Th17-type T-cell response is implicated during immune response against numerous enteric pathogens and has importance in regulating commensal bacteria in lumen and gut homeostasis but its role in immune response against *L. intracellularis* has not been fully elucidated. IFNγ is an important cytokine in cell-mediated immunity and it could be secreted by different cells in intestinal mucosa, not just $CD4^+$ or $CD8^+$ T cells. It was reported that IFNγ could be secreted by Th cell population that has a mixed Th1/Th17-type phenotype and therefore the Th1/Th17-type phenotype could be implicated in a pathogenic immune response and clinical outcome from disease. Further, because intestinal epithelial and innate immune cells express TLR2, 4 and 5 (ligands of which are present on *L. intracellularis*), it must be determined experimentally whether the respective agonists impact the response to *L. intracellularis* infection or pathogenesis. Molecular mechanisms present in bacteria could play an important role in regulating host immune response but secreted or other molecular factors have not yet been reported. IgA plays important role in protection against enteric pathogens, regulation of commensal bacteria, and intestinal immune homeostasis. Although increased titres of IgA are present in the intestinal mucosa of *L. intracellularis* infected pigs from day 15 to 29 dpi, the extent of protection mediated by IgA is still unknown (285, 7) and needs to be clarified.

1.3.5 Immune protection against *Lawsonia intracellularis* infection

(Modified version of this chapter was used to write review article "Immune response and protection against *Lawsonia intracellularis* infections in pigs", Obradovic M. and Wilson L. H.; submitting to Veterinary Immunology and Immunopathology)

There are two reported ways to achieve complete immune protection against *L. intracellularis* infection: re-inoculation of pigs with virulent bacteria 10 weeks after primary infection or vaccination of animals with live, avirulent vaccine or inactivated vaccine (288, 289, 283, 287, 290). It was shown that pigs infected orally with 10^5 virulent or 10^{10} avirulent *L. intracellularis* developed PPE and resolve infection on their own (288). After 10 weeks, when pigs stopped shedding bacteria in their feces, animals were re-challenged orally with 10^{10} bacteria and clinical symptoms such as elevated titres of serum antibodies and fecal shedding were monitored (288). Re-challenged animals did not show signs of clinical disease characteristic for PPE nor was fecal shedding of bacteria detected by PCR; elevated serum IgG antibodies were detected in most of the re-challenged pigs (288). Thus, pigs previously infected with *L. intracellularis* were protected from subsequent challenge for at least 10 weeks with no clinical signs or bacterial shedding detected regardless of initial dose of bacteria (288).

A more recent study using age-matched pigs in the experimental challenge confirmed these findings that prior infection protects against subsequent (289). Pigs infected with intestinal mucosa containing 10^{10} *L. intracellularis* by the oral route then treated with antibiotics followed by oral challenge with approximately 10^{10} *L. intracellularis* at day 49 after the first infection were referred to as re-challenged pigs. Pigs infected for the first time with 10^{10} *L. intracellularis* on day 49 were referred to as challenge control pigs. Uninfected and unchallenged pigs were also used as control

animals. Re-challenged pigs showed lack of fecal shedding of *L. intracellularis*, low levels of acute phase protein concentrations in serum and low levels of bacterial antigen in the intestinal mucosa comparable to the challenge control pigs. However, challenge control pigs shed *L. intracellularis* in feces and showed extensive *L. intracellularis* antigen within all layers of the intestinal mucosa, and significant acute phase protein response in serum after the experimental infection relative to uninfected control pigs (289). In the challenge control group, serum concentrations of C-reactive protein and haptoglobin were increased from 6 dpi, and increased serum concentrations of haptoglobin were generally observed 2–3 weeks after primary infection both at 5–6 and 12–13 weeks of age (289). Re-challenged pigs had a significantly lower increase in haptoglobin and C-reactive protein then what was observed in challenge control pigs, which may reflect the observed protection and lack of clinical disease in re-challenged pigs (289). Acute phase proteins are non-specific indicators of disease and inflammation and theirs lower levels detected in re-challenged pigs may imply that adequate adaptive immune response was able to protect animals without eliciting a strong inflammatory response. This study is the first to report elevated acute phase proteins in response to *L. intracellularis* infection and should be investigated as a correlate of protection in future vaccine trials (289).

The same group published the follow-up a study where they evaluated the cell-mediated and humoral immune responses in pigs following primary and secondary challenge exposure to *L. intracellularis* (284). They confirmed former findings that the previously infected pigs were protected against oral re-infection with bacteria (284). After a second exposure to *L. intracellularis*, animals did not show characteristic clinical symptoms of PE, bacteria were not detected in feces and there was a significant reduction in the pathology of intestinal mucosa compared to pigs only infected once (284). A second exposure to bacteria initiated stronger

memory cell-mediated immune response compared to the primarily infected pigs and the dominant IFNγ producing cells were characterized as $CD8^+$ and $CD4^+CD8^+$ double positive lymphocytes (284). Results of this study imply that protective immunity against *L. intracellularis* may be mediated by $CD8^+$ effector cells and $CD4^+CD8^+$ double positive memory T-cells (284).

Currently, there are two registered vaccines against *L. intracellularis* available to prevent of PE in pig farms. The first registered vaccine was an avirulent live bacteria developed by multiple consecutive passaging of the bacterial isolate B3903 in McCoy cells (291), (Enterisol®, Boehringer Ingelheim). The attenuation of virulence was achieved between 20 and 40 passages *in vitro* which is represented by attenuation and down-regulation of important genes responsible for virulence and cell metabolism in bacteria (74). The commercial vaccine administered orally to weaners and each dose is comprised of 10^5 bacteria (291). One study showed that after challenge with virulent bacteria, vaccinated pigs showed partial protection with a significant reduction in intestinal lesions, absence of clinical symptoms, and higher average daily gains compared to the unvaccinated control group (291). Although fecal shedding was reduced in the vaccinated group (47% and 40% reduction on day 35 and 42, respectively), vaccinated animals still shed bacteria in great numbers and therefore may be a source of transmission of *L. intracellularis* in naïve pigs (291). The potential reason for this partial immune protection by the oral vaccine may be due to the low dose recommended by the manufacturer that may not be sufficient to induce strong immune response when administered to animals in drinking water. Indeed, a study published by Nogueira et al 2013 compared prescribing the recommended oral dose ($10^{4.9}$ $TCID_{50}$) to 10-times oral ($10^{5.9}$ $TCID_{50}$) and the recommended dose administered via the i.m. route (283). Pigs that received 10-times higher oral dose were protected after challenge to a higher level than animals that received the other doses (283). Pigs orally vaccinated with 10-times dose showed increased serum and mucosal

concentrations of IgM and IgG antibodies, increased TNF-α and TGFβ1 in the intestinal mucosa, and a trend towards higher levels of serum IFNγ and IL-6 on day 17 (283). Levels of serum IgG titres were increased between day 9 and day 17 for all vaccinated groups and those levels were similar for 1x dose oral and i.m. vaccinated animals (283). In contrast, serum and mucosal levels of IgA titres were below detection limits in all vaccinated groups (283). To test the protective properties of oral and i.m. vaccination against *L. intracellularis*, vaccinated and control animals were challenged orally with 25 ml of a suspension containing around 10^9 bacteria total (283). Vaccination with 1x oral dose induced modest serum and mucosal-scrapings IgG titres but vaccination with a 10-times dose resulted in higher titres of *L. intracellularis* specific IgG titres in the first two weeks after vaccine administration (283). After challenge, the control vaccinated animals had increased serum cytokines levels of IFNγ, IL-6, IL-10 and TNF-α from day 0 to day 21 post-challenge and a significant increase in serum *L. intracellularis*-specific IgG and IgA (283). Although all vaccinated pigs in this trial had significantly reduced fecal shedding of *L. intracellularis* after virulent challenge compared to the non-vaccinated control group, animals vaccinated with 10-times dose shed significantly fewer bacteria in their feces and had less total percentage of ileum affected than the other two vaccinated groups (283). The observed immune protection of oral 10-times dose might be attributed to stronger priming of both cell-mediated and humoral immunity in intestinal mucosa than a 1x oral dose or 1x i.m. dose. Intramuscular administration of 1x dose of live avirulent vaccine was also able to provide protection from virulent challenge, as determined by reduced fecal shedding and intestinal pathology (283). This suggests that serum antibodies might play role in preventing entry of bacteria into the cells or by ADCC (283). The immune correlates of protection after virulent challenge could not be determined in this trial due to the high variability of serum cytokines levels of IFNγ, IL-6, IL-10, and TNF-α in

vaccinated and control pigs and the absence of correlation between cytokine levels and severity of clinical symptoms (283).

A recent study explored immune correlates of protection by comparing immune responses and level of protection after a *L. intracellularis* challenge of orally vaccinated pigs and pigs previously infected with bacteria (287). Both humoral and cellular mediated immune response in vaccinated animals were low compared to animals infected with virulent bacteria. Oral vaccination with 10^5 bacteria per dose did not induce elevated antigen-specific IgG and IFNγ levels in serum which was in contrast to the previous study of Guedes and Gebhart were they reported seroconversion and increased levels of serum IFNγ after oral vaccination (287, 48). These data indicate that dose and/or strain dependent induction of immune responses might play a critical role in eliciting robust or modest immune responses (283).

In the second part of the study by Riber et al. 2015, control, vaccinated, and primary infected animals were challenged with a virulent *L. intracellularis* strain (287). The vaccinated and control animals shed high levels of bacteria in their feces while no shed bacteria were detected in feces of primary infected animals (287). These results confirmed previous findings of incomplete protection and shedding of bacteria in animals vaccinated with the 10^5 oral vaccine dose (291, 283). In addition, re-challenged animals had significantly higher induction of antigen-specific proliferation of $CD4^+CD8^+$ and $CD8^{high}$ $CD4^{neg}$ T cells compared to vaccinated group (287). Although the IFNγ response was increased in both vaccinated and control groups after challenge, vaccinated animals showed induced proliferation of IFNγ producing $CD4^+CD8^{neg}$ T cells after challenge whereas $CD8^+$ and $CD4^+CD8^+$ produced IFNγ after challenge in previously infected pigs (287).

This difference in the induction of cell-mediated immune response was suggested to be the main reason for different levels of protection observed between different groups. One the one hand, 1x dose of oral vaccine was not sufficient to mount complete protection against virulent challenge and prevent bacterial shedding in feces. On the other hand, vaccinated animals showed no symptoms of PE, showed significantly less intestinal mucosal pathology and significantly higher weight gain than non-vaccinated animals after challenge with a virulent strain. Secondary exposure was sufficient and able to provide complete immune protection presumably due to activation of cellular immune response mediated by IFNγ producing $CD4^{+}CD8^{+}$ and $CD8^{high}$ $CD4^{neg}$ T-cells (287). These findings suggest activation of memory T-cell in intestinal mucosa that promptly react to *L. intracellularis* invasion and elicit a strong CMI. Further, differences in immune response between orally vaccinated and re-challenged pigs reported in several studies may exist due to a low dose of vaccine given in drinking water and due to loss of important immune stimulant antigens during attenuation of the vaccine strain (283, 287). Increases levels of intestinal IgG, IgM, IgA, TGFβ and IL-10 observed after challenge in animals that received the 10-times oral vaccine dose may contribute to the clearance of bacteria and promote resolution of infection (283). The local humoral immune response and elevated secretion of suppressive cytokines may play an important role in protection by controlling inflammation and promotion of restoration of the epithelial barrier. Taken together these data indicate that an ideal vaccine should be able to elicit balanced immune response where both cell-mediated and local humoral immunity should work in concert to eliminate *L. intracellularis* and control excessive inflammation.

The second commercially available vaccine against *L. intracellularis* contains inactivated whole cell bacteria in an oil-in-water emulsion and adjuvant based on mineral oil and alpha-tocopherol (Vitamin E) (290), (Porcilis Ileitis®, Merck Animal Health, Medison, NJ, USA).

Protection was assessed under field conditions when the vaccine was administered i.m. to 22-25 days old pigs immediately prior to weaning (290). The major findings from this study were that the vaccine-induced significant serum antibody titres and upon subsequent challenge, vaccinated pigs showed a 15-fold reduced bacterial fecal shedding and bacterial burden and microscopic lesions in ileum relative to the control group (290). Vaccinated animals had preserved gut integrity and preserved goblet cells as detected by unaltered secretion of molecular marker MUC2 and positive Periodic Acid Schiff (PAS) staining (290). This study demonstrated that animals vaccinated i.m. with the inactivated vaccine showed reduced incidence of disease and severity of clinical symptoms of PE as well as reduced colonization and duration of fecal bacterial shedding in response to challenge (290). Studies need to be undertaken to determine whether the immune correlates of protection include induction of humoral immune response (283, 290).

In modern pig husbandry, vaccination timing is crucial to achieving protection of the herd. Serological tests play important role in seroprofiling and timing of vaccination in naïve animals. Results showed that crucial period for infection risk is in the nursery and that optimal period for vaccination is between 5 and 8 weeks (292).

1.4 ANTIGEN SELECTION FOR SUBUNIT VACCINE DEVELOPMENT

1.4.1 Bioinformatics and reverse vaccinology

Vaccines are considered one of the most important public health measures in the prevention of both human and animal diseases. The conventional or classical development of vaccines relied on biochemical and microbiological methods of isolating, growing, attenuating or killing of pathogens and administration of pathogen with or without adjuvants to induce immune protection in animals or humans. These methods have changed little since their inception by Dr. Jenner in 1796 (293), as many pathogens that could be cultivated in the laboratory and that had low antigen variability could trigger a protective humoral immune response (294, 293). One notable exception to this rule is a vaccine against human hepatitis B virus where the virus cannot be cultured in the lab but high titres of the virus can be isolated from infected persons and used to formulate the vaccine (295). Examples of successful and protective conventional vaccines include bacterial vaccines against tetanus, pertussis, viral-like polio, rabies, and others. Conventional vaccines succeeded in the eradication of one human disease, smallpox (296), and one animal disease, rinderpest (297). Polio has been very nearly eradicated where incidence has decreased more than 99 % since the late 1980s (298). Despite this success, conventional design of vaccines has failed to develop effective vaccines for many important human and animal pathogens that display antigenic diversity, that cannot be cultured *in vitro*, that lack adequate animal models, and that require inducing cell-mediated or mucosal immunity to achieve immune protection (299).

Development of sequencing technology and bioinformatics during the 1990's made available new tools for exploring microorganisms, which were also applicable in vaccine development. Sequencing of the bacterial genome of *Haemophilus influenzae* in 1995 marked the

turning point in the methodology used to explore antigenic characteristics of microorganisms, which in subsequent years initiated advances in vaccine development (300). It then became possible to use information from genome sequencing coupled with bioinformatics analysis to screen for potential antigens present in microorganisms without requiring their cultivation or requiring time-consuming and laborious microbiological techniques to isolate and purify antigens in quantities adequate for vaccine formulation (301). This new antigen discovery methodology for vaccine design was named reverse vaccinology (301).

The term reverse vaccinology was first proposed by Dr. Rappuoli who identified a process of antigen discovery using *in silico* analysis of pathogen genome sequences using bioinformatics and producing recombinant proteins that could be subsequently screened for antigenic and immunogenic properties (301). This method was appropriately named because antigen selection starts from the pathogen genome and not from an organism itself such as in conventional vaccinology. Reverse vaccinology allows for the screening of hundreds of proteins coded in the genome for their capability to induce an immune response and to produce antigens (302). The prime example of successful application of reverse vaccinology principles is the development of a vaccine against *Neisseria meningitidis* serogroup B (MenB). Conventional vaccinology was not able to develop a vaccine against MenB due to similarity of the MenB capsule polysaccharide with human polysialic acid. This high degree of similarity resulted in low immunogenicity, high variability of antigens across MenB strains, and poor cross-protection (303, 304). Sequencing of the complete genome of virulent MenB strain led to the identification of 2158 open reading frames (ORFs) (305). The search for new potential antigens of MenB started by screening the whole MenB genome and 570 ORFs were predicted to code for surface or secreted proteins (306). A total of 350 ORFs were found suitable for cloning in *Escherichia coli* and recombinant proteins were

purified, tested for surface localization by FACS and ELISA analysis. The antigens were selected based on whether these recombinant proteins were recognized by antibodies and whether they could promote protective immunity (306). After completion of this selection process, 90 previously unknown antigens were discovered, of which 28 were able to induce antibodies (Abs) that killed the bacteria (306). In contrast, previous studies using the conventional methodology to fraction bacterial extracts for vaccine antigen discovery yielded only 12 MenB surface antigens, of which only four induced antibodies with bactericidal activity (302). Five antigens identified by reverse vaccinology approach were selected for vaccine formulation: *Neisseria* antigen 1870 (GNA1870; which is factor H–binding protein [fHBP]), GNA1994 (which is NadA), GNA2132 (GenBank accession number NP_275117), GNA1030 (GenBank accession number AAF41429), GNA2091 (GenBank accession number NP_275079), and outer membrane vesicles (OMV) from the New Zealand MeNZB vaccine strain, which contains the immunogen PorA (307, 299). The final vaccine formulation consisted of a fHBP-GNA2091 fusion protein, a GNA2132-GNA1030 fusion protein, NadA, and OMV and this vaccine which was registered under the name 4CMenB and acquired market authorization for the European Union in January 2013 (299, 298). The 4CMenB vaccine was tested in clinical studies in European countries on a large number of children and results showed excellent immunogenicity, safety, and compatibility with pediatric vaccines given at the same age (308, 309). Promising results from clinical studies in Europe prompted approval of 4CMenB in other countries, including Australia, Canada, UK and USA (310). To achieve adequate protection and protective antibodies titres in children, different vaccination schedules and doses were proposed. The general conclusion was that infants should receive two to three doses before the end of their first year of life and one booster dose after the first year to keep protective antibodies titres high (310). Children between 2-10 years should receive two doses in

not less than 2 months intervals and adolescents and adults should receive 2 booster immunizations with the interval not less than one month (310). The 4CMenB vaccine was also successfully applied as a preventive measure during two outbreaks at Princeton and Santa Barbara Universities in the USA caused by two different clones of MenB (310). Development, clinical trials, successful implementation in vaccination schedules of 4CMenB and application for use as a preventive measure during outbreaks of meningococcal diseases have demonstrated the enormous potential of reverse vaccinology to provide a solution for prevention of bacterial diseases in humans.

Reverse vaccinology has also been used to develop new vaccines against animal diseases such as contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* (Mmm), an important disease in sub-Saharan African countries. Reverse vaccinology identified 66 candidate proteins that were ranked based on recognition by serum antibody titers from CBPP-positive cattle (311, 312). The best candidates were used as vaccines in animal challenge trial wherein groups of animals were immunized with five different proteins. Two groups of animals were protected from the *Mmm* challenge with one group showing complete clearance of the *Mmm* from lung samples (312). In a third group, there were a reduced number of reported animals with lung lesions and *Mmm* could not be cultured from lung samples (312). Although immunization with the recombinant proteins was able to protect animals after challenge, the study also reported that some combination of proteins used for immunization actually increased immune-related pathology after the challenge (312). These findings indicate the importance of adequate testing of vaccine recombinant protein candidates both in *vitro* and *in vivo* experiments to select antigens that can confer protective immunity.

With the development of sequencing and bioinformatics, more data on microorganism genomes have become available which has led to a new branch of reverse vaccinology named pan-

genomic analysis (313). The pan-genomic analysis compares genomes across all strains belonging to the certain specific phylogenetic clade. It defines two subsets of genomic repertoire, the core genome (i.e. genes shared across all strains of the clade) and dispensable genome (i.e. genes that are specific to subsets of strains) (298). This comparative analysis of the genomes applied to multiple pathogenic isolates of *Streptococcus agalactiae* led to the discovery of significant interspecies gene diversity and identification of bacterial proteins suitable for vaccine formulation to test for protection against multiple strains (313, 314). The comparative analysis of the complete genome sequences of eight *S. agalactiae* strains coupled with bioinformatics yielded 589 genes encoding proteins predicted to be expressed on the surface or secreted proteins (314). Of these proteins, 396 belonged to the core genome and 193 belonged to the indispensable genome (314). After 312 proteins were expressed in *E. coli*, soluble recombinant proteins were screened as suitable vaccine antigens for their ability to confer immune protection in the infant mice model of disease (314). After extensive testing, four proteins, one from the core genome and three from the dispensable genome were selected as vaccine candidates that could elicit protection against all eight circulating strains of *S. agalactiae* (314). The importance and utility of pan-genome approach were also proved in genome analysis of multiple strains of *S. pneumoniae* which led to the identification of genes that encode three previously unknown proteins that form subunits of two distinct pili (315). Antibodies raised against these proteins enabled the use of immune-electron microscopy to image bacteria and discovery of two types of pneumococcal pili (PI-1 and PI-2) (315). Pili are abundant and long filamentous structures protruding from the bacterial surface. Because different strains have different types of pili, the global genomic approach was successfully used to identify the frequencies of two types of pili and to suggest that a vaccine against multiple strains should be formulated to include antigens from both types of pili (316). Additional screening

for other vaccine antigen candidates conserved among different strains should be conducted in order to achieve comprehensive coverage of all circulating *S. pneumococcal* strains. The pan-genomic approach offers great possibilities for screening potential vaccine candidates across multiple strains and selecting the best one based on conferred immune protection against all virulent strains in circulation.

Although classical and structural vaccinology have not fulfilled the promise of developing protective vaccines against HIV-1, reverse vaccinology principles were successful in developing effective vaccines against some human and animal viral diseases. A vaccine against Hepatitis B virus registered for human use, RECOMVIVAX HB, is based on recombinant HBsAg protein produced in yeast cells and formulated with aluminum adjuvant (Merck. http//merck.com) (298). Recently, two vaccines against human papillomavirus (HPV) were approved by the US Food and Drug Administration (298) made from different HPV recombinant proteins produced in yeast or insect cells formulated with immune enhancers and adjuvants (317). They show great safety and protection against HPV mediated cervical cancer (317). In veterinary medicine, a vaccine against West Nile virus registered for use in horses consists of a combination of existing canarypox vaccine with genes expressing two proteins from West Nile virus (318, 298). With the improvement of bioinformatics and a growing understanding of host-pathogen interactions, new vaccines and therapeutics against infectious or other diseases, such as cancer, autoimmune or neurodegenerative diseases, could be developed using reverse vaccinology methods.

Application of reverse vaccinology methodology does not require growing pathogens *in vitro* and does not require inactivation or attenuation of the pathogen. Thus, vaccines developed by reverse vaccinology are safe and eliminate the risk of reversion to virulence which is a risk associated with live attenuated vaccines (298). By using bioinformatics, we can avoid regions of

the antigens which may be potentially toxic, allergy-causing or potentially oncogenic. Subunit vaccines developed by reverse vaccinology are economical and easily scalable which appeals to the pharmaceutical needs but also offers great efficacy and flexibility for Public Health system during infectious diseases outbreaks. Less than two decades of reverse vaccinology has resulted in important scientific discoveries and applicable vaccine solutions for a limited number of diseases. Future predicted development in bioinformatics and improved molecular techniques has overcome many previous vaccine development limitations and lead to development of new vaccines and immune therapeutics for a wide range of diseases that currently lack adequate prophylactic or therapeutic measures.

Although reverse vaccinology has been applied successfully in the development of vaccines for bacterial and some viral diseases, both in humans and animals, this approach is not without its own limitations. Imperfect bioinformatics prediction programs and systemic validation including complicated and incomplete *in-silico* prediction of discontinuous epitopes, difficulties using *in-silico* approaches to predict how sequences translate to functional proteins and whether post-translation modifications on the recombinant proteins are critically required are all limitations to the reverse vaccinology approach. Other functional limitations are that there are only a small number of protein expression systems available to express the recombinant proteins, and each protein may have their own difficulties in expression and purification and solubilization of recombinant proteins with their natural conformation, which may be critical for immune recognition. Disease-specific difficulties may include challenges in comparing multiple isolates of pathogen species for multi-strain vaccine protection, incomplete knowledge on the impact of host-pathogen interactions on protein conformation, and lack of known immune correlates of protection for certain diseases. Finally, genetic diversity of the host as well as challenges to discern the ideal

route of administration, challenges in overcoming mucosal tolerance for mucosally-administered vaccines and the choice of appropriate adjuvants all impact vaccine development that may not be overcome simply by using reverse vaccinology approaches (301, 306, 314, 319, 299, 302).

In addition to reverse vaccinology and pan-genomic selection of antigens, certain bacterial proteins could be subjected to in-depth structural analysis by X-ray crystallography or other techniques to identify 3D structures. Knowledge of the 3D structure of proteins may lead to further advancement in the formulation of bacterial subunit vaccines by selecting antigens that can better mimic pathogenic protein shape. This approach was named structural vaccinology in the field of bacterial vaccines (320). A subunit vaccine formulated to include these proteins may prevent bacterial invasion and thus propagation of intracellular bacteria. Structural vaccinology has been expanded to identify HIV-1 proteins that could be used as vaccine against HIV (302). It investigated the structure of viral proteins and their importance in immune invasion and as possible targets for neutralizing antibodies (321). Although important information on protein and antibodies structure were gained, structural vaccinology in HIV-1 research did not lead to success in identification of neutralizing antibody targets that could be used in a vaccine (302). The reasons for this failure may lie in viral capacity to evade immune responses during infection, the complicated nature of immune recognition and unknown immune correlates of protection for HIV-1 infection. Structural vaccinology has been applied to both viral and host immune system protein structures that are involved during the HIV-1 infection and we anticipate new discoveries that could lead to effective HIV vaccines or therapies. Proteins expressed on the pathogen surface that are critically required for invasion of the host may be suitable vaccine targets.

1.4.2 2-D gel electrophoresis to identify proteins targeted by host immune system.

2-DE is an efficient analytical tool for separation of complex proteins mixtures from tissue, mammalian and bacterial cells, as well as their secretions (322). The commonly used workflow for detection of proteins from complex mixtures includes sample preparation or extraction, protein separation by 2-DE, protein gel staining, identification, characterization, and quantitation of proteins with Electrospray ionization Liquid chromatography–mass spectrometry (ESI LC-MS/MS) followed by virtual or experimental 2-DE protein database construction (323). The basic principle of 2-DE is the separation of proteins in two dimensions. The first dimension is the separation of proteins based on their isoelectric point (pI) and the second dimension is separation depending on molecular mass (MW) of proteins (322). Both separation steps must have very good resolution and can to resolve up to 5,000 protein spots with high degree of accuracy (324). A particular advantage of 2-DE analysis and separation power is the ability to visually detect protein post-translation and co-translation modifications, which are currently impossible to accurately predict using a genomic approach (322). To visualize the resolved protein spots, a gel is stained with Coomassie brilliant blue or silver nitrate (323). Coomassie staining is less sensitive (can detect ng levels) than silver staining (can detect pg levels) but it has better linearity, accuracy and is more compatible for downstream MS analysis (323). The use of formaldehyde as a reductant during the developing stage in silver staining could cross-link proteins, which hampers MS analysis (325). To overcome this problem, a formaldehyde-free silver staining was developed which enhanced compatibility with downstream MS applications (326). In addition to these commonly used stains, modern proteomics developed improved methods for protein detection with a high agreement with MS, such as using fluorescent stains and colloidal Coomassie blue (G250 and R250) (327, 328, 329).

2-DE is a robust and proven technique that is compatible with other biochemical techniques (330) such as Western blot and MS and has been used to detect antigens from bacteria (331, 332), cancer cells (333) and fungi (334) that are recognized by the human immune system. The resolution and sensitivity of 2-DE is such that it has been used to analyze proteins that have not been characterized by other methods, such as nucleolus proteins or secreted proteins from myeloid cells secretome (335, 336, 337). Bacterial cells are excellent candidates for 2-DE analysis due to their lower complexity compared to mammalian cells which are an essential requirement for successful protein separation (330). However, limitations of 2-DE include difficulty in precise reproduction of gels and difficulties in separating proteins that are hydrophobic, very acidic or basic (322). To achieve highly reproducible gels, it may be advisable to lower the complexity of the sample by analyzing specific regions of the bacterial or secreted bacterial products. Bacterial culture growth conditions must be constant to avoid metabolic adaptations that could lead to a change in protein expression that could contribute to sample variability (330). The problem of low reproducibility of IEF was mitigated by the development of immobilized pH gradient (IPG). This commercially available pre-casted acrylamide gel matrix co-polymerized with a pH gradient on a plastic strip has many advantages over traditional ampholyte method (322). The main advantages are the capacity to circumvent cationic accumulation, production of better-focused protein without smearing, reduced cathodic drift, higher mechanical strength, higher protein loading capacity and commercial availability of different pH ranges and sizes of the IPG strips (322). With the improvement of IEF, improved staining of gel and downstream analysis such as MS and the use of image analysis software, the only bottleneck left in the 2-DE protocol is sample preparation. It is widely acknowledged that the major weakness of 2-DE protocol is the separation of hydrophobic and highly acidic proteins, especially low abundant, low molecular weight membrane proteins

(330). The problem emerges due to insufficient extraction and solubilization of hydrophobic or acidic proteins in IEF buffers (322) which has been somewhat improved with development of improved IEF compatible detergents (338, 339, 340, 341). Despite improved solubilization of membrane proteins with detergents, effective detection of membrane proteins still remains the greatest challenge for 2-DE analysis (330).

WB analysis and MS add to the analytical power of 2-DE and allows accurate identification of targeted proteins. For example, 2-DE followed by MS lead to identification of 310 antigenic protein from *Helicobacter pylori*, 32 of which were bound by hyperimmune serum generated against *H. pylori* (342). Out of these 32 antibody targets, nine were newly identified antigens whereas 23 were previously confirmed antigens (342). Another study used 2-DE immunoproteomics to identify membrane antigens of uropathogenic *E. coli* expressed during urinary tract infection (343). This study identified 23 outer membrane antigens, together with a novel iron compound receptor and antigens that play important roles in UPEC pathogenesis, such as ChuA, IroN, IreA, Iha, IutA, and FliC (343). 2-DE coupled with MS was used to identify interactions between membrane complexes of *E. coli* which yielded 31 proteins with some newly discovered interacting proteins (344). This approach identified bacterial outer envelope proteins, which can then be functionally proven to play a role in the bacterial membrane, highlighting the versatility of this method (344). The capabilities of 2-DE to identify important outer membrane proteins and potential antigens from *Staphylococcus aureus* (345), *Streptococcus thermophilus* (346), extraintestinal pathogenic *E. coli* (347) and other bacteria (331, 332). These studies exemplify the technical capabilities of 2-DE coupled with MS to identify important proteins in the outer membrane of pathogenic bacteria which could be selected as promising antigens for vaccine formulation.

We have adapted 2-DE to resolve *L. intracellularis* proteins to identify proteins recognized by antibodies from hyperimmune serum and to determine which of these immunogenic protein bind to host cells. We predicted that proteins that fit these criteria may be neutralizing antibody targets which were then validated experimentally.

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

We hypothesize that serum antibodies specific for *L. intracellularis* can be used to identify antigenic bacterial proteins suitable for subunit vaccine development.

2.2 Objectives

The main objective is to use functional biological assays to identify proteins in *L. intracellularis* which are the targets of neutralizing antibodies. The identified antigens can then be used in a subunit vaccine to promote strong neutralizing antibodies in pigs.

Objective 1. Developing an assay approach using flow cytometry and qPCR to evaluate if antibodies against *L. intracellularis* can block *L. intracellularis* penetration of eukaryotic cells and subsequent growth, *in vitro*.

Objective 2: Using modified 2-D electrophoresis and Mass Spectrometry to identify proteins unique to *L. intracellularis* that are recognized by rabbit hyperimmune serum.

Objective 3: Perform *in silico* screening of identified proteins to predict which ones are expressed on the bacterial surface followed by cloning of selected proteins into *E. coli* to produce recombinant proteins.

Objective 4: *In vitro* evaluation of inhibitory capabilities of sera against recombinant proteins on *L. intracellularis* penetration into porcine intestinal epithelial cells. Compare our approach to select Bioinformatics Platforms and evaluate recombinant antigen characteristics and potential to formulate subunit vaccine.

3. USE OF FLOW CYTOMETRY AND PCR ANALYSIS TO DETECT 5'-CARBOXYFLUORESCEIN-STAINED OBLIGATE INTRACELLULAR BACTERIA *LAWSONIA INTRACELLULARIS* INVASION OF MCCOY CELLS

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Relationship of this study to the dissertation

L. intracellularis is obligate intracellular pathogen that has been difficult to precisely quantify during infection. Also, evaluation of neutralization properties of sera or other immune components on bacterial entrance into the cells have not been investigated completely due to the lack of adequate *in vitro* invasion and quantification assays. Thus in this study, we devise a method to quantify invasion of obligate intracellular bacteria, *L. intracellularis,* inside McCoy cells and a method to establish whether antibodies neutralize bacterial adherence or invasion of eukaryotic cells. The results of this study suggest that coupling CFSE-labeling of bacteria and qPCR analysis can be utilized to detect and quantify bacterial invasion and may be a useful tool for studying the invasion of eukaryotic cells by intracellular bacteria.

3.1 Introduction:

L. intracellularis are obligate intracellular Gram-negative bacteria that cause an infectious enteric disease known as PE or ileitis. This bacterium is endemic in intensive swine herds all over the world (5). PE is a major cause of weight loss and mortality in pigs and represents a significant economic burden for the swine industry (6, 47) and it is an emerging pathogen in horses (348). In the 1970's, G.H.K Lawson's group identified bacteria in proliferative lesions in the pigs suffering from PE but it proved extremely challenging to isolate and characterize these bacteria (349). It was not until 1993 that the same group managed to isolate these bacteria, establish the *in vitro* growth conditions and confirm that these bacteria were responsible for this disease (31, 4). The bacteria were finally characterized and classified as *L. intracellularis* and their role in the disease was established through oral inoculation of pigs (350, 351). The isolation and *in vitro* cultivation of *L. intracellularis* is still difficult and studying these bacteria in laboratory and field condition is limited by current tools and techniques.

It remains very challenging to detect, track and quantify obligate intracellular bacteria if antibodies are not available. Labeling extracellular bacteria with CFSE has been useful in tracking the presence and activity of environmental and food-borne microorganism (352, 353, 354, 355, 356, 357, 358). CFSE staining proved a suitable method for identifying *Chlamydia trachomatis* elementary bodies in human monocytes (359). Because CFSE has minimal effects on bacterial adhesion, viability, and metabolic activity, and it is readily retained in bacteria for long periods, we predicted it may be useful for quantifying invasion or cellular association of *L. intracellularis* within its host cell. This work was undertaken to critically evaluate whether labeling *L. intracellularis* with CFSE can be used to develop an easy and effective high-throughput method to track and quantifying infected eukaryotic cells. Coupled with qPCR analysis and confocal

microscopy, we confirmed that the bacteria invaded the host cell and were not merely cell adherent. Use of fluorescence activated cell sorting (FACS) allowed for sorting of infected from non-infected cells for further experimentation. To show that this approach was compatible with functional analysis, we introduced hyperimmune serum and measured the impact that antibodies had on bacterial invasion and growth. These simple techniques can be adapted for use with other obligate intracellular pathogens and are amenable to be used for a myriad of functional analysis.

3.2 Material and methods

3.2.1 Animals and hyperimmune serum.

All animal experimental procedures were performed in accordance with the Procedures for Ethics Review of Animal Use Protocols and approved by the University Committee on Animal Care and Supply (UCACS), University of Saskatchewan.

Two female New Zealand White rabbits (2-3 kg weight) were kept in isolation units in the Animal Care Unit of VIDO-InterVac. To generate rabbit hyperimmune serum, rabbits were injected via the intramuscular route with the Enterisol® vaccine (Boehringer Ingelheim, Burlington, ON) which is comprised of live avirulent *L. intracellularis* strain. The vaccine consisted of 20 mg of Enterisol® was dissolved in 500 µl of vaccine sterile diluent and 500 µl Incomplete Freund's Adjuvant (Sigma-Aldrich, Oakville, ON). Each rabbit was vaccinated on day 0, 21, and 42. Rabbit hyperimmune sera were collected via exsanguination following euthanasia of the rabbits (Euthanyl, Bimeda-MTC Animal Health INC., Cambridge ON, Canada) 60 days after the first vaccination. All blood samples were collected using Ethylenediaminetetraacetic acid

(EDTA) Vacutainers (BD Biosciences-Canada, Mississauga, ON) followed by centrifugation (2500 × *g*) before the serum was stored at −20 °C until use.

3.2.2 Cell culture and bacterial propagation conditions.

Mouse fibroblast cells (McCoy B, ATCC CRL-1696) were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Burlington, ON) with 10 % Fetal Bovine Serum (FBS; Gibco Life Technologies, Burlington, ON) in a humidified incubator in an atmosphere of 5 % CO_2 and 95 % air at 37 ºC in media with 10 µg/ml gentamycin (Bio Basic Canada Inc., Markham, ON) and 1 % L-Glutamine (Gibco #25030-081). Cells were passaged two times per week in 1:5 ratio in Corning 75 cm^2 cell culture flasks.

Prior to infection with *L. intracellularis*, cells were cultured in their respective medium without FBS or gentamycin for 24 h at 37℃ in a mixture of gases (10 % hydrogen, 10 % carbon dioxide and 80 % nitrogen gas) in zip lock bags (99). Cells were then co-cultured with *L. intracellularis* (from the Boehringer-Ingelheim's Enterisol® avirulent live vaccine suspended in vaccine sterile diluent as directed) at a MOI of 0.1 with a cell density of 1 x 10^5 cells per well. The number of avirulent *L. intracellularis* in the vaccine was calculated based on data published by Guedes et al. (48), that one milliliter of the Enterisol® vaccine contains 5.3 x 10^5 organisms.

3.2.3 CFSE-labelling procedure for *L. intracellularis* and Flow Cytometric Analysis.

The CellTrace™ CFSE Cell Proliferation Kit for flow cytometry (C34554, Molecular Probes, Life Technologies) was used to stain live avirulent *L. intracellularis* with CFSE according to manufacturer's instructions. The CFDA-SE form is non-fluorescent and passes through cell membranes of prokaryotic and eukaryotic cells. Once inside cells, CFDA-SE is converted to

fluorescent CFSE which is well-retained within the bacteria, even after several days in a cell culture environment (360). *L. intracellularis* from Enterisol® vaccine were rehydrated with 1 ml of vaccine sterile diluent then centrifuged at 6,000 x g for 5 min and suspended in 500 µl of PBS (Gibco Life Technologies, # 20012-027). The bacteria were incubated with 1 µl of 5 mM CFSE in 500 µl of PBS and incubated at 37 ºC for 10 min. Next, 500 µl of FBS (Gibco Life Technologies) was added and the samples were incubated for an additional 15 min at room temperature to quench the CFSE reaction. The CFSE-stained bacteria were then washed three times with PBS and centrifuged at 6,000 x g for 5 min, to remove excess dye. CFSE-labeled bacteria were re-suspended in DMEM for infection of McCoy cells.

To ensure that the CFSE was retained in the bacteria and did not leak into the culture supernatant resulting in staining of the eukaryotic cells, we adapted the following procedure from Vander Top et al (361). Briefly, CFSE-stained bacteria were suspended in tissue-culture cell-specific media as indicated above for 1 h at 37 ºC. CFSE-bacteria were removed by centrifugation at 6,000 x g for 5 min and the remaining supernatant was incubated with $0.1x10^6$ McCoy cells at 37 ºC for 1 hr. After this time, the cells were centrifuged and then suspended in PBS plus 2% FBS.

Flow cytometric analysis was performed using a BD FACS Calibur™ flow cytometer (BD Biosciences). We detected CFSE fluorescence in the FL1 channel, with gating being selected based on uninfected, unstained McCoy cells as our negative control cells. Flow cytometer results were analyzed in Kaluza software (Beckman-Coulter).

3.2.4 Cellular penetration assays.

To assess the penetration of bacteria into the cells, CFSE-stained live-avirulent *L. intracellularis* were used to infect McCoy cells that were plated 24 h previously at a density of (0.1×10^6 cells/per well/24 well plate). An MOI of 0.1 CFSE-stained *L. intracellularis* was used to infect each well. During the infection, the McCoy were cultured in their respective media without FBS and gentamycin for 24 hr at 37 ℃ in mixture of gases (10% hydrogen, 10% carbon dioxide and 80% nitrogen gas) in ziplock bags (99). After 24 hr of incubation, cells were trypsinized and collected for flow cytometric analysis.

3.2.5 Neutralization assays.

Rabbit serum was complement-inactivated with heating 56℃ for 30 min. The Bicinchoninic acid (BCA) analysis (Pierce BCA Protein Assay Kit, #23225, Thermo Scientific) was performed to quantify protein concentration. Protein concentration of pooled rabbit serum from vaccinated animals was 108.5 mg/ml. Protein concentration of pooled rabbit serum from control non-immunized animals was 53.8 mg/ml.

The CFSE-stained avirulent *L. intracellularis* were incubated in media without FBS or gentamycin for 60 min at room temperature in 1.5 ml Eppendorf tubes with increasing volume of complement-inactivated rabbit serum (ratio of bacteria to serum was 1:0.1, 1:1 or 1:2 as indicated). As detailed above, McCoy cells previously seeded onto a 24 well plate were infected with CFSE-stained bacteria (MOI 0.1) bound by rabbit antibodies. The cells and bacteria were left to incubate 24 hr at 37℃ in zip lock bags filled with ma ixture of gases detailed above. After 24 hr, cells were trypsinized and collected for analysis by flow cytometry.

3.2.6 PCR analysis to detect *L. intracellularis*-specific DNA in the neutralization assay.

McCoy cells were seeded to an MOI of 0.1 bacteria with or without pre-incubation with rabbit serum detailed above. After 1, 3 or 5 days, cells were trypsinized and resuspended in DMEM Complete media containing 10% FBS (Gibco Life Technologies) to arrest the trypsin reaction. Cells were pelleted by centrifugation at 500 x g for 10 minutes. The pellet was suspended in NaOH buffer (25 mM NaOH, 0.2 mM EDTA) and heated for 95℃ for 1 hr to rupture the cells and release the DNA from the nucleus. HCl (40 mM Tris-HCl, pH 5.5) was added to neutralize the solution. Primers designed against amino acid ABC transporter substrate-binding protein (GlnH, Locus LI0754) from the *L. intracellularis* PHE/MN1-00 genome (Forward: 5'-GGTTAGTCGTTGCCCATGATA-3', Reverse: 5'-CTGCGATATGCTCCCATAGTT-3') were used to quantify genome copy number. Quantitative real time PCR (qPCR) was conducted using Kapa Syber Green Mastermix (Kapa Biosystems, Wilmington, MA) with data collected using a Step One Real-Time PCR System (Applied Biosystems by Life Technologies).

3.2.7 FACS, cytospin, and fluorescent confocal microscopy.

McCoy cells were seeded into T-75 tissue culture flask ($1x10^6$ cells per flask) in DMEM with 10% FBS (Gibco Life Technologies) with 50 μg/ml gentamycin (Bio Basic Canada, Inc.) and 1% L-glutamine (25030-081; Gibco Life Technologies) in a humidified incubator (5% CO_2 and 95% air at 37ºC). After one day of growth and around 50% confluency, cell growth medium was replaced with medium without antibiotics and FBS, cells were infected at a MOI 0.1 or CFSE-labelled *L. intracellularis* and the flasks were incubated as detailed above. After 24h after infection cells were trypsinized, centrifuged at 500 x g for 10 minutes and resuspended in FACS sorting

buffer (PBS, 1mM EDTA, 25mM HEPES pH 7.0, 1% FBS heat inactivated, filtered through a 0.2 mm filter and stored at 4 ^{o}C). Cells were sorted into $CFSE^{-}$ and $CFSE^{+}$ on a MoFlo sorter (Beckman Coulter, Indianapolis, IN) (362). $CFSE^{-}$ and $CFSE^{+}$ cells were stained with PKH26 as per manufacturer's instructions (Mini26-1kt, Sigma). PKH26 stained cells, 10,000 per fraction, were collected on microscope slides using Cytospin 4 (Thermo Fisher Scientific) at 1,000 rpm for 3 min, dried and protected with ProLong Gold antifade reagent with DAPI (Molecular probes, Life Technologies) before covering with cover slips. Fluorescent microscopy was performed after 24 h using Leica SP8 confocal microscope (Leica Mycrosystems Inc., Concord, ON, Canada).

3.2.8 Western blot analysis

Whole cell *L. intracellularis* proteins or McCoy cell proteins were subjected to 10% SDS-PAGE gel and analysed by WB using rabbit serum from animals vaccinated against avirulent *L. intracellularis* or rabbit serum before immunization (negative control).

Proteins were transferred by a semi-dry transfer protocol to a nitrocellulose membrane (BIO-RAD, 162-0094) using Bio-Rad Trans-Blot SD Semi-Dry transfer cell (10 V for 30 min) and then WB analysis was performed using hyperimmune serum (1:500; obtained from rabbits immunized with whole bacteria) or rabbit serum before immunization (negative control) as primary antibodies. Anti-rabbit IR 800 antibody (1 µg/ml; Li-COR, Lincoln, NE, USA) was used as secondary antibody. The membrane was scanned with Odyssey scanner (Li-COR®) in the IR 700 and IR 800 channels.

3.2.9 Statistical Analysis.

One way ANOVA with Kruskal-Wallis test was used to compare flow cytometry and qPCR data and medians were compared using Dunn's test. All statistical analyses and graphing were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Differences were considered significant if $p<0.05$.

3.3 Results and Discussion

3.3.1 Detection of CFSE-stained *L. intracellularis* in eukaryotic cells using flow cytometric analysis.

L. intracellularis is an intracellular bacterium 0.1 to 0.3 μm in width and 0.7 to 2.0 μm in length (1), which is sufficiently large to be detected using flow cytometric analysis We stained *L. intracellularis* with lipid-permeable fluorescent molecule carboxyfluoroscein diacetate (CFDA)-succinimidyl ester (CFDA-SE). Once inside bacteria or eukaryotic cell, the diacetate groups are cleaved leaving fluorescent carboxyfluoroscein. Fuller et al., showed that CFSE efficiently stained Gram positive and Gram-negative bacteria without causing undesirable effects on cell adhesion or viability (355).

Unstained and CFSE-stained *L. intracellularis* were subjected to flow cytometric analysis. A representative forward (FSC-H) and side scatter (SSC-H) dot plot and the gating of viable cells areshown in Figure 3.1. The side-scatter on the y-axis is an indicator of the cellular granularity/complexity of *L. intracellularis* and the forward-scatter is an indicator of size. Unstained bacteria were introduced to the flow cytometer; events within Gate A are live, unstained *L. intracellularis* (Fig 3.1A) which indicates that these bacteria are indeed of sufficient size and

complexity to be quantified using flow cytometry. The population indicated by the arrow (Fig 3.1A) represent dead bacteria or cellular debris, which were not included in the gate and therefore excluded from further analysis. Next, CFSE-stained bacteria were introduced to the flow cytometer and an identical gate was used to quantify the percentage of events (i.e. the percentage of bacteria) that share similar same size and complexity as was observed in the unstained bacteria (Fig 3.1B). As expected, we did not see a shift in the percentage of events outside of Gate A, which confirms that the CFSE stain does not radically impact bacterial size or structure complexity. Because the CFSE-staining procedure has multiple washes, any cellular debris have been washed away from the live bacteria, which explain why the population indicated by the arrow in fig. 3.1A is not present in fig. 3.1B. We measured fluorescence in the FL1-H channel along the y-axis to detect CFSE and the results are shown in figures 3.1C and 3.1D. A gate was drawn above the background fluorescence of the unstained bacteria (Fig. 1C) and this identical gate is then used to measure fluorescence of the CFSE-bacteria (Fig 3.1D). As expected for this negative control, only negligible fluorescent events are detected in the unstained bacteria (0.31%; Fig. 3.1C). When the identical gate was introduced to the CFSE-stained *L. intracellularis*, we observed that 99.36% of the bacteria were fluorescent in the FL1-H channel (Fig 3.1D). These results indicate that we can detect *L. intracellularis* using flow cytometric analysis and that our CFSE staining procedure was efficient in staining the majority of bacteria.

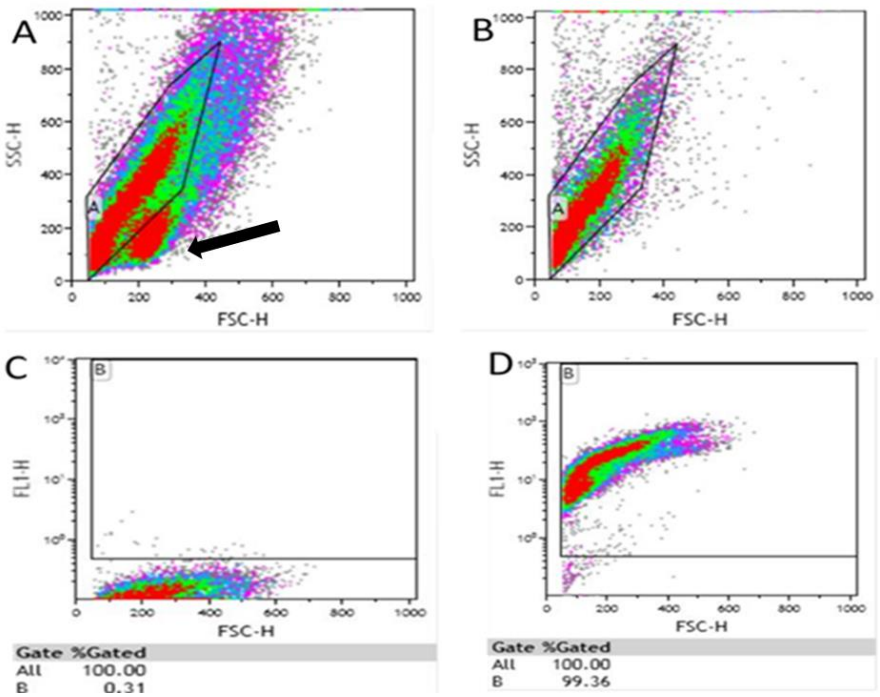

Figure 3.1 Flow cytometry analysis of avirulent *L. intracellualris* non-stained forward/side scatter plot (A), CFSE stained forward/side scatter plot (B), non-stained forward scatter/FL1 channel plot (C) and CFSE stained forward scatter/FL1 channel plot (D). Experiment was done three times.

3.3.2 Quantification of invasion or cellular association of CFSE-labelled *L. intracellularis* into McCoy cells

McCoy cells are routinely used for *L. intracellularis* propagation and growth in laboratory and industrial environments (363) and therefore we used flow cytometry to assess invasion and/or cellular adherence of *L. intracellularis* with McCoy cells. Figure 3.2A shows the flow cytometric gating strategy for live McCoy cells. Because McCoy cells are collected using trypsin and centrifugation (explained in detail in Materials and Methods) any free bacteria, not associated with the cells were removed and Gate A includes only viable McCoy cells. Figure 3.2B shows the fluorescence for mock-infected McCoy cells which served as a negative control and, as expected, showed negligible fluorescent events (0.4%) in FL1-H gate. These data confirm that McCoy cells

are not auto-fluorescent in this channel. Also, as another negative control, we analyzed McCoy cells infected with non-stained a-virulent bacteria gated with same parameters as previous. Again our control showed negligible fluorescent events (0.4%) in the FL1-H gate (data not shown). McCoy cells incubated with a MOI of 0.1 CFSE-stained bacteria showed that 21.4 % of the McCoy cells were fluorescent indicating invasion by and/or cellular association with CFSE-stained bacteria (Fig. 3.2C). McCoy cells incubated with a MOI of 0.3 CSFE-bacteria showed that 50.4 % of McCoy cells were invaded by or associated with CFSE-bacteria (Fig 3.2D). In all subsequent experiments, a MOI of 0.1 CFSE-stained bacteria were used to infect McCoy cells. The average percentage of McCoy cells infected with CFSE-stained bacteria was 19.5% +/- 5.2 (Fig 3.2E).

Next, we confirmed that the CFSE dye was retained inside *L. intracellularis* and that our results were not compromised by CFSE leaking out of the bacterial cells and staining McCoy cells. The CFSE-labeled bacteria were suspended in cell media for 1 h at 37 ^{o}C and then the bacteria were removed by centrifugation (361). McCoy cells were then cultured in this medium that had previously contained the CFSE-stained bacteria with the idea being that any CFSE that had leaked out of the bacteria could penetrate the McCoy cells. We observed that despite being cultured in a medium which previously contained CFSE-stained bacteria, the McCoy cells showed negligible fluorescence in the FL-1 channel (0.3%; Fig 3.2F) confirming that the CFSE stain did not leak from the bacteria and stain the eukaryotic McCoy cells.

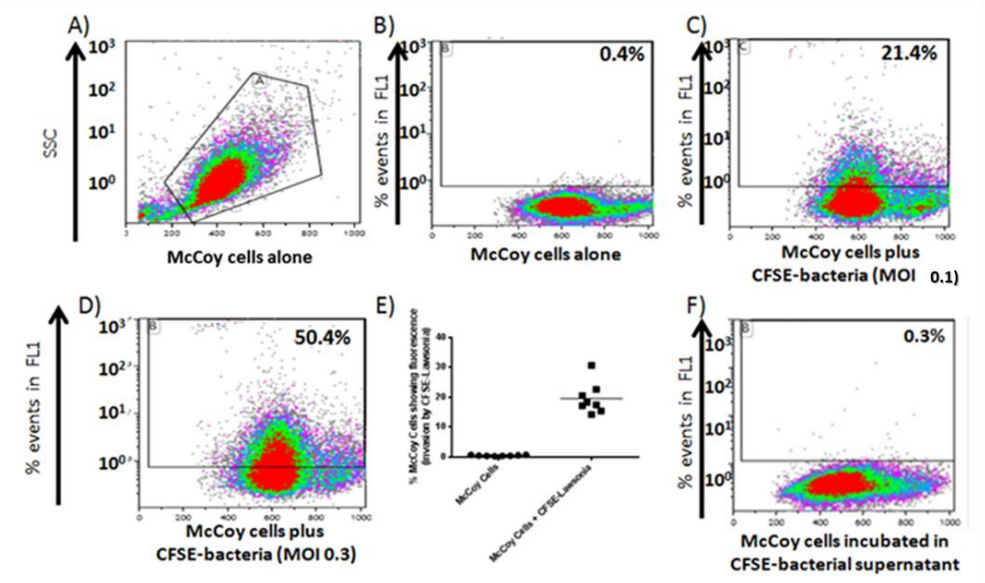

Figure 3.2 Flow cytometry analysis: gating strategy on live McCoy cells forward/side scatter plot (A), gating strategy on live McCoy cells forward scatter/FL1 channel plot (B), McCoy cells infected with CFSE labeled *L. intracellularis* MOI 0.1 (C), McCoy cells infected with CFSE labeled *L. intracellularis* MOI 0.3 (D), multiple biological replicates are shown on one graph (E) and McCoy cells incubated in CFSE-bacterial supernatant forward scatter/FL1 channel plot (F).

We concluded that flow cytometry can be used to quantify fluorescence attributable to CFSE-stained bacteria in solution (Fig 3.1) or after invasion or adherence to McCoy cells (Fig 3.2). Because the CFSE stain did not leak from the bacteria and the gating is specific for McCoy cells (i.e. it will not include any free CFSE-stained bacteria), fluorescence in FL1-H detected in McCoy cells must be attributed to CFSE-stained bacteria that are adherent to or have invaded the McCoy cells.

3.3.3 Establish bacterial invasion of McCoy cells using PCR analysis and fluorescent microscopy.

Because *L. intracellularis* are obligate intracellular bacteria, they can only replicate if they have invaded a susceptible eukaryotic cell. Thus if the qPCR analysis indicates that McCoy cells infected with bacteria show increased bacterial DNA over time, we indirectly establish that the growing bacteria are intracellular. We infected McCoy cells with *L. intracellularis* (MOI 0.1) and performed qPCR analysis on McCoy cells 1, 3 and 5 days after infection (Fig 3.3). As expected, mock-infected McCoy cells showed no increase in bacterial DNA over time. However, after McCoy cells were incubated with *L. intracellularis* for 1, 3 and 5 days, we observed a significant increase in fold change of bacterial DNA relative to the bacterial DNA present on day 1 (($p<0.05$ and $p<0.001$, respectively). For each biological replicate, we observed growth over time (indicated by lines joining data points). We conclude that because the fold change in bacterial DNA is increasing over time, these obligate intracellular bacteria have invaded the McCoy cells and are growing.

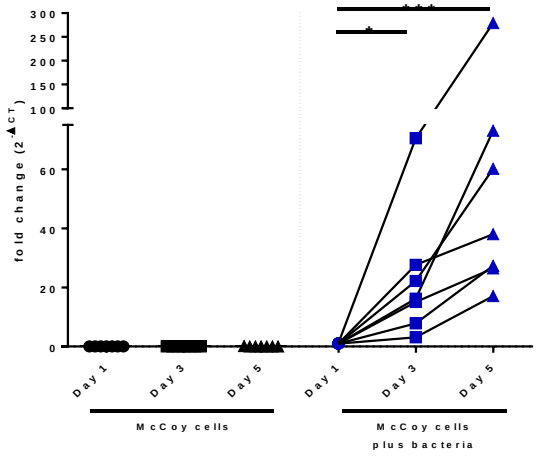

Figure 3.3 qPCR analysis of McCoy cells and McCoy cells infected with avirulent *L. intracellularis* Day 1, Day 3 and Day 5. Fold change of bacterial DNA relative to the bacterial DNA present on day 1 ((*) day 3 $p<0.05$ and (***) day 5 $p<0.001$). Data present five independent experiments for each time point.

We next used fluorescent confocal microscopy to directly confirm that *L. intracellularis* invaded the eukaryotic cells and were not merely adherent to their surface. We incubated McCoy cells with CFSE-stained bacteria for 24 h as indicated above but this time we performed fluorescent activated cell sorting (FACS) which allowed us to physically separate the fluorescent McCoy cells (i.e. the cells that were invaded by the $CFSE^+$ bacteria) from the non-fluorescent McCoy cells (i.e. McCoy cells that were not invaded by the labeled bacteria). We plated the uninfected (data not shown) and infected McCoy cells on microscope slides and stained the cells with DAPI to fluorescently label the nuclei (bright white colour in the first panel of figure 3.4 and indicated by blue colour; in the last panel). Also, we stained the cell membrane with PKH26 which is shown as grey and white in the second panel and red in the last panel of figure 3.4. The $CFSE^+$ bacteria are present as bright white spots in the 3rd panel of figure 3.4 and green in the last panel of Figure 4. The uninfected McCoy cells did not show green fluorescence which confirms that the cells are not autofluorescent and that they are negative for $CFSE^+$ bacteria (data not shown). The infected McCoy cells have green scattered flecks or inclusions in the cytosol and are not co-localized with

the DAPI or PKH26 stain. These images confirm that the bacteria have invaded the McCoy cells and not adherent to the cell membranes nor are they localized to the nucleus.

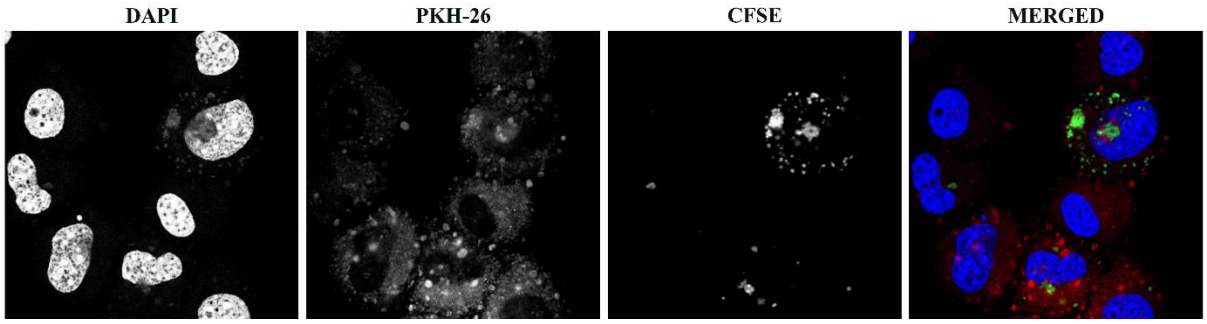

Figure 3.4 Fluorescent confocal microscopy of infected McCoy cells. McCoy cells sorted for CFSE positive signal (green), cytospinned and stained with PKH26 (red) and DAPI (blue).

3.3.4 Neutralization assay to assess whether serum antibodies blocked bacteria invasion of McCoy cells.

To determine whether humoral immunity could contribute to immune protection, we investigated the potential impact that serum antibodies had on the invasion of *L. intracellularis* into McCoy cells and the bacterial growth. The role of antibody-mediated immunity to mediate protection against intracellular microorganisms is still a debated topic (364). Pigs with PE consistently show an accumulation of IgA within ileal epithelial cells (349, 13, 365) and there is some evidence that serum antibodies may be sufficient to generate protective immunity against *L. intracellularis* (366). Others have shown that antibodies may mediate their protective immunity against PE partly by coating the bacteria so that once they are within the enterocytes, the antibody-pathogen complex could undergo proteasomal degradation via antibody-dependent intracellular neutralization (ADIN) (367).

We repeatedly immunized rabbits with the Enterisol® vaccine (consisting of 10^5 *L. intracellularis*) and obtained their serum after 60 days. To confirm that the antibodies in the hyperimmune serum have specificity for *L. intracellularis*, we performed total protein isolation from *L. intracellularis*, McCoy cells (which are known to be present in the vaccine strain) as well as McCoy cells infected with *L. intracellularis*. We subjected the total proteins to SDS-PAGE electrophoresis, transferred the proteins to a membrane and hybridized the membrane with hyperimmune serum or control rabbit serum (Fig. 3.5). Lane 1 shows fractionated bacteria and McCoy cells. Antibodies in the hyperimmune serum bound to many proteins in this lane. Lane 2 shows fractionated McCoys cells and serum from the immunized rabbits failed to bind McCoy cell proteins. Lane 3 shows fractionated bacteria and McCoy cells hybridized with control rabbit serum and no proteins were bound. Thus, we are confident that the rabbit hyperimmune serum has antibodies specific for *L. intracellularis*.

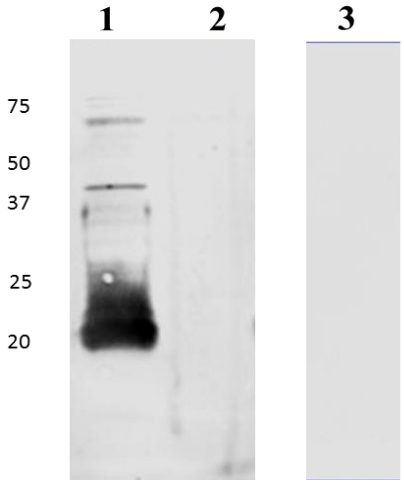

Figure 3.5 Western blot analysis of *L. intracellularis* protein fractions from whole cell isolation method: 1- total protein isolation samples from a-virulent *L. intracellularis* detected using positive rabbit serum; 2- total protein isolation from McCoy cells detected using positive rabbit serum, 3- Total protein isolation from avirulent *L. intracellularis* detected with negative rabbit serum. Anti-rabbit antibodies were detected with an IRDye 800 nm conjugated goat-anti-rabbit antibody.

To investigate whether CFSE-labelling of bacteria can be used in conjunction with functional assays, we investigated whether antibodies in hyperimmune serum could impact bacterial invasion and/or bacterial growth. Flow cytometry was performed on the McCoy cells infected with bacteria alone or with CFSE-stained bacteria pre-incubated with 1/10th volume (1:0.1 volume), an equal volume (1:1) or twice the volume (1:2) rabbit hyperimmune serum to bacteria. The CFSE-stained bacteria were then introduced to cultures of McCoy cells. As expected, McCoy cells cultured alone were negative for fluorescence (Fig 3.6A) and McCoy cells cultured in the presence of CFSE-stained bacteria showed that approximately 17.1% of cells were fluorescent (i.e. infected). When 1:0.1 volume of rabbit serum was incubated with the bacteria prior to infection, we observed that approximately 27.8% of cells were fluorescent. When the volume of rabbit serum incubated with the bacteria prior to infection was increased to 1:1 or 1:2, we observed approximately 38.4% and 40.4% fluorescent cells, respectively. Significantly more CFSE-stained bacteria pre-incubated with 1:1 rabbit hyperimmune serum invaded the McCoy cells ($p<0.05$) relative to the bacteria not preincubated with the serum. In contrast, when we incubated the CFSE-bacteria with negative rabbit immune serum, we did not observe increased events in FL1-H (19.3%) (Fig 3.6B). These results indicate that rabbit anti-*L. intracellularis* antibodies in positive rabbit serum enhanced bacterial invasion of McCoy cells.

Finally, we performed qPCR analysis to establish whether pre-incubating bacteria with rabbit hyperimmune serum impacted bacterial replication. As detailed in figure 3.6, the qPCR analysis was performed 1, 3 or 5 dpi of McCoy cells with *L. intracellularis* alone or pre-incubated with rabbit hyperimmune serum (Fig. 3.6C). After 5 days, bacteria incubated with McCoy cells in the absence of rabbit serum showed significantly increased fold change in bacterial DNA relative to day 1 ($p<0.001$). When 1:1 or 1:2 volume of rabbit serum was preincubated with the bacteria,

we observed significantly more bacterial DNA present after 5 days ((p<0.01), (p<0.05), respectively). These data indicate that the bacteria were replicating inside McCoy cells despite pre-incubation with rabbit antibodies.

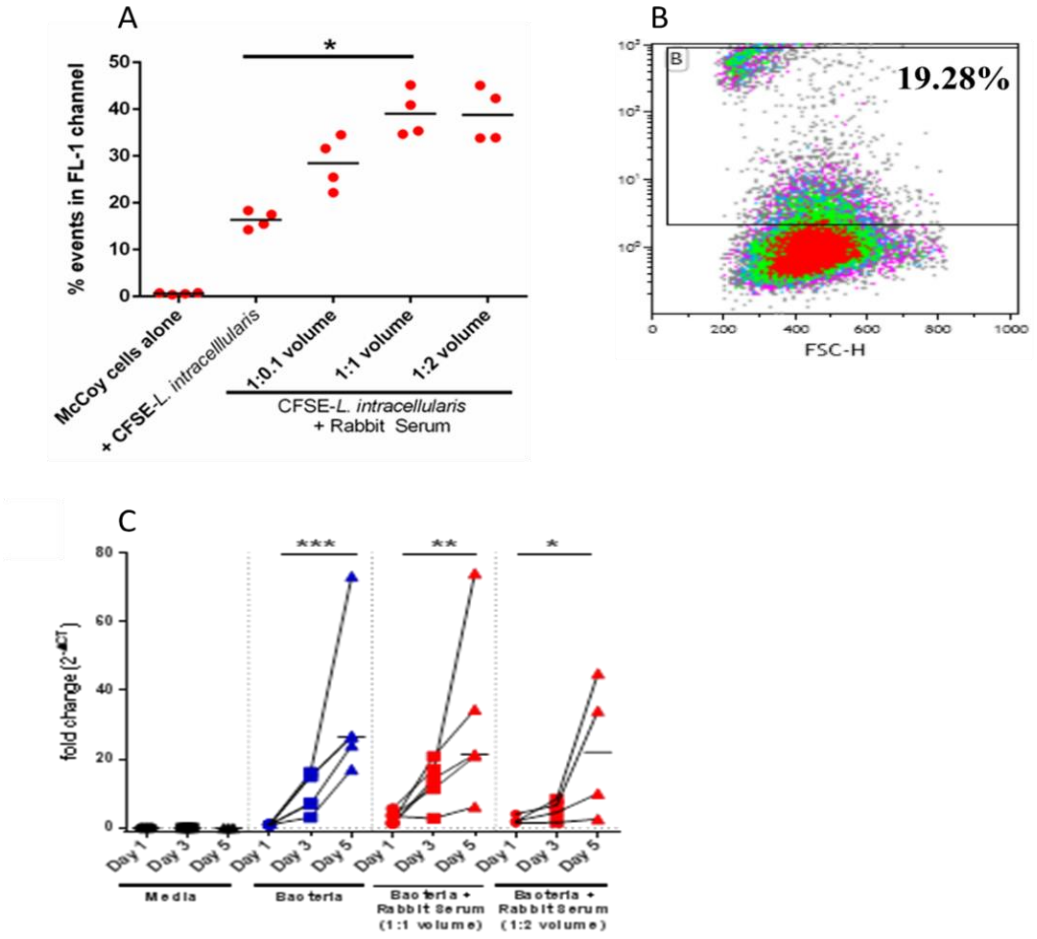

Figure 3.6 Flow cytometry and qPCR data from neutralization assay with increasing volume of positive serum. Multiple flow cytometry data (n=4) from neutralization assay with increasing volume of positive rabbit serum (A), forward scatter/FL1 channel plot of McCoy cells infected with CFSE labeled *L. intracellularis* (B) and qPCR analysis of McCoy cells, McCoy cells infected with avirulent *L. intracellularis* and McCoy cells infected with avirulent *L. intracellularis* incubated with rabbit serum Day 1, Day 3 and Day 5 (C). Fold change of bacterial DNA relative to the bacterial DNA present on day 1 bacteria alone ((***) p<0.001, (**) p<0.01 and (*) p<0.05).

Using these techniques, we determined that rabbit hyperimmune serum antibodies promote bacterial invasion into McCoy cells.

3.4 Conclusions:

The present data show that CFSE fluorescent dye can stain Gram-negative *L. intracellularis*, that the CFSE fluorescent stain is stable and retained inside the bacteria, and it does not significantly affect bacterial viability and growth. Using flow cytometric analysis, we showed that CFSE-stained *L. intracellularis* infected McCoy cells in culture in an oxygen-reduced gas environment (Fig 3.2). Benefits to using CFSE labeling of obligate intracellular microorganism are numerous. CFSE staining of bacteria is cheap and easy to perform and CFSE-stained bacteria can be detected inside the host cells without fixation and permeabilization of the eukaryotic cells. Unlike antibodies that bind to bacterial surface proteins which may be important for adhesion or invasion, CFDA-SE stain is permeable to the lipid bilayer and therefore does not bind surface proteins. CFSE-stained bacteria can be sorted from uninfected eukaryotic cells via cell sorting without the use of antibodies or beads bound to the surface that may impact function. Sorting can be used to establish eukaryotic and/or bacterial differential gene and protein expression and other changes in cellular events during adhesion and infection. By coupling qPCR analysis with fluorescent microscopy, we confirmed that the bacterial invaded the host cell were replicating and were not merely cell adherent. CFSE-staining of bacteria was compatible with functional analysis and rabbit hyperimmune serum promoted bacterial uptake and growth. These simple techniques can be adapted for use with other obligate intracellular pathogens and are amenable to be used for a myriad of functional analysis.

Student contribution: M.O. performed the flow cytometric analysis, established the growth conditions for avirulent *L. intracellularis* in the tissue culture cell lines, tissue culture analysis, PCR, neutralization assay, microscopy experiments, designed experiments and assisted in the writing of the manuscript.

4. IMMUNOPROTEOMIC ANALYSIS OF *LAWSONIA INTRACELLULARIS* IDENTIFIES CANDIDATE NEUTRALIZING ANTIBODY TARGETS FOR USE IN SUBUNIT VACCINE DEVELOPMENT

(Submitted for a provisional patent application and to Veterinary Microbiology for publication)

Relationship of this study to the dissertation

Due to its obligate intracellular nature, characterization of *L. intracellularis* antigens and proteins involved in host-pathogen interaction and immune recognition have not been elucidated using conventional microbiological techniques. In this study, we utilized 2-dimensional gel electrophoresis coupled with Western-immunoblotting, mass spectrometry, and bioinformatics to identify proteins on the surface of bacterium that interacts *in vitro* with pig intestinal cells (IPEC-1) and that have immunogenic properties. Using this methodology we were able to detect 11 immunogenic bacterial proteins from which four were predicted to be expressed on the outer membrane. Recombinant proteins were produced from *E. coli*, purified using affinity chromatography. Western blot analysis determined that these recombinant proteins were immunogenic as they were recognized by porcine hyperimmune serum specific for whole *Lawsonia* lysate. In chapter 3 we described the use of CFSE stained *L. intracellularis* for evaluating anti *L. intracellularis* sera neutralization properties. This neutralization assay was applied to evaluate neutralization properties of rabbit hyperimmune sera generated against vaccine strain of *L. intracellularis* and sera specific for each recombinant protein. Our results indicate that each recombinant protein is a potential neutralizing antibody target and candidate for subunit vaccine formulation.

4.1 Introduction

Attachment of *L. intracellularis* to enterocytes is an important step in bacterial infection but the mechanism by which these bacteria interact with the host cells has not yet been determined (3). Proteins which comprise the Type III secretion system (T3SS), a common secretion system found in many enteroinvasive pathogens that plays the role in invasion and suppression of innate defenses, has been detected in three *Lawsonia* isolates (368). These T3SS proteins and other uncharacterized bacterial proteins that facilitate contact with enterocytes are potentially important immunogens as they are expressed on the surface and therefore accessible to the host immune system. Establishing that these proteins are important for attachment has been hampered by the obligate intracellular growth requirement of *L. intracellularis* and by difficulties of removing eukaryotic host cell proteins from sample preparation. Modern proteomic analysis coupled with a fully sequenced and partially annotated *L. intracellularis* genome offers the means to identify potential antigens. For example, the *L. intracellularis* autotransporter protein (LatA) was detected by Watson et al 2011 using mass spectrometry (MS) and bioinformatics (103). The same group applied shotgun proteomic analysis to identify 19 unique proteins during *in vitro* infection of which two proteins, LI0841, and LI0902, were shown to have antigenic properties (104). The success and efficacy of proteomic analysis to detect yet uncharacterized *L. intracellularis* proteins prompted us to perform 2-dimensional gel electrophoresis (2-DE) coupled with WB analysis to identify antigens that could be detected by hyperimmune serum and be used towards the formulation of neutralizing subunit vaccine.

2-DE is an efficient analytical tool for separation of complex proteins mixtures from tissue, mammalian and bacterial cells, and secretions (322). 2-DE is a robust and confident technique with the advantage of being compatible with other biochemical techniques (330). For instance, 2-DE

coupled with WB and MS has been used to detect antigens from bacteria (331, 332), cancer cells (333) and fungi (334) that are recognized by the human immune system. Bacterial cells are good candidates for 2-DE analysis due to their lower complexity compared to mammalian cells, which is an essential requirement for successful protein separation (330). A downstream application such as WB and MS add to the analytical power of 2-DE and allows efficient identification of targeted proteins.

To detect *L. intracellularis* immunogenic proteins that play a role in adherence or invasion, we combined the separation power of 2-DE with WB and MS. The resulting target genes were then cloned, and proteins expressed and evaluated for their immunoreactivity and capacity to interfere with bacterial-host interactions under competitive conditions. Our analysis revealed 11 unique *L. intracellularis* proteins and further bioinformatics analysis and flow cytometry assay indicated that four were predicted to be possible vaccine targets.

4.2 Materials and methods

4.2.1 Cell culture conditions

Undifferentiated porcine intestinal epithelial cell lines (IPEC-1), which were derived from the jejunum and ileum of unsuckled one day old piglets (369), were cultured and maintained in DMEM/ F-12 (SH30271.01; HyClone, Thermo Fisher Scientific, San Jose, CA, USA) with 5% Fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada), penicillin/streptomycin (Gibco 5000 units/ml Penicillin, 5000 μg/ml Streptomycin), insulin (10 μg/ml), transferrin (5.5 μg/ml), selenium (5 ng/ml) (ITS; Sigma-Aldrich) and 5 ng/ml of epidermal growth factor (Sigma-Aldrich).

Cells were kept in a humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37 ^oC and passaged two times per week at 1:5 ratio in Corning® 75 cm^2 cell culture flasks. IPEC-1 cells used for *L. intracellularis* infection and neutralization assays were grown as indicated above but in the absence of antibiotics.

4.2.2 *L. intracellularis* protein sample preparation

L. intracellularis pellets prepared from infected McCoy cells (detailed in (4)) were suspended in RIPA buffer (0.05 M Tris pH 8, Bio Basic Canada INC, Markham. ON, Canada); 0.15 M NaCl (Bio Basic Canada INC.); 0.10% SDS, (Bio Basic Canada INC.); 1% Deoxycholic acid, (VWR-Amresco, Dublin, Ireland); 1% Nonidet P40 substitute (Sigma-Aldrich); distilled water) complete with 0.1 M phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) in isopropanol (Sigma-Aldrich). The sample was freeze/thawed three times to rupture the bacterial cells and the mixture was centrifuged at 10,000 x g for 10 min. Bacterial proteins were then precipitated from the supernatant with ice-cold acetone. The mixture was vortexed and stored in -20 °C for 1 h then the incubation mixture was centrifuged 14 000 x g for 10 min. The supernatant was carefully discarded and the pellet dried before resuspending with $NaHCO_3$ buffer and quantifying by BCA analysis following the manufacturer's instructions (Pierce, Thermo Fisher Scientific).

The *L. intracellularis* proteins were labeled with Cy5 dye (GE Healthcare Life Sciences-Amersham Biosciences, Mississauga, ON, Canada) in a dye/protein molar ratio of 8:1, following the manufacturer's recommended protocols. The mixture was incubated for 4 h at room temperature in the dark. Unbound dye was removed by size filtration using 3000 MWCO 15 ml

filters (Millipore, Etobicoke, ON, Canada) with four additional washes with ultra-pure water. The final concentration of Cy5-labelled *L. intracellularis* proteins was determined by BCA assay (Pierce) prior to 2-DE.

4.2.3 Binding of Cy5-labeled *L. intracellularis* proteins to IPEC cells

IPEC-1 cells were grown to confluence in T-75 flasks trypsinized and washed three times with antibiotic and FBS-free IPEC medium. Next, $1x10^6$ IPEC cells were incubated with 700 μg of Cy5-labeled bacterial proteins for 3 h with gentle mixing on nutator at 4 ^oC. Cells were centrifuged as indicated above and the unbound *L. intracellularis* proteins were removed with the supernatant. The IPEC-1 cells and bound *L. intracellularis* proteins were then suspended in RIPA buffer with PMSF (Sigma-Aldrich) and subjected to repeated freeze/thaws as indicated above. IPEC-1 proteins and Cy5-labeled adherent *L. intracellularis* proteins were then subjected to 2-DE.

4.2.4 Two dimensional gel electrophoresis

Proteins from lysed IPEC-1 cells and bound Cye-5 labeled *L. intracellularis* (250 μg used for the analytical gels and 600 μg for preparation gels) were solubilized in rehydration buffer overnight (9 M urea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS, Fisher BioReagents), 1% DL-Dithiothreitol (DTT , Promega, Madison, WI, USA), 2% pharmalyte 5-8 (GE Healthcare), 0.002% bromophenol blue (BioRad, Hercules, CA, USA) and were loaded onto an IPG strip (Immobiline ^{tm} DryStrip, pH 4-7, 13 cm, GE Healthcare). The strips were individually subjected to IEF using the IPGphor device (GE Healthcare-Amersham

Biosciences) following the stepwise protocol (150 V step and hold for 3h, 300 V step and hold 1200 Vh, 1000 V gradient for 3900Vh, 8000 V gradient for 13500 Vh and 8000 V step and hold for 25000 Vh). After IEF, both IPG strips were stored at -80 ^{o}C. The IPG strips with isoelectric focused proteins were thawed at room temperature and equilibrated with SDS equilibration buffer with 1% DTT (Dithiothreitol) (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue) for 15 min at room temperature followed by washing with SDS equilibration buffer with 2.5 % Iodoacetamide (GE Healthcare) for 15 min. After equilibration, strips were placed over SDS gels and covered with sealing solution (0.5% agarose in 1x SDS running buffer). Second dimension electrophoresis was performed using BIO-RAD protean® II xi Cell Apparatus and two medium sizes, 10 % SDS PAGE gels. Electrophoresis was performed using 90 V constant voltage for 16 h with constant water cooling of apparatus (Bio-Rad Power pack 200).

4.2.5 Western blot analysis and silver staining

Proteins on the analytical gel were transferred with semi-dry transfer to a nitrocellulose membrane (BIO-RAD, 162-0094) using Bio-Rad Trans-Blot® SD Semi-Dry transfer cell (15 V for 60 min) and then Western blot analysis was performed using rabbit hyperimmune serum (1:500; obtained from rabbits i.m immunized with avirulent vaccine strain) as primary antibodies. Anti-rabbit IR 800 antibody (1µg/ml; Li-COR®, Lincoln, NE, USA) was used as secondary antibody. The membrane was scanned with Odyssey scanner (Li-COR) in the IR 700 and IR 800 channels. IR800-stained proteins indicate bacterial proteins with affinity for IPEC-1 cells and bound by rabbit serum against whole bacteria (See Figure 4.1 A).

For the preparative gel, *L. intracellularis* proteins were stained with Silver stain kit, (PROTSIL-1-KT, Sigma Aldrich) according to manufacturer's protocol and this gel was reserved for excising gel spots for MS analysis (See Figure 4.1 B).

4.2.6 Preparation of samples for Mass Spectrometry

Silver-stained proteins on the preparative gel which correspond to IR-800 labeled proteins detected by WB analysis were excised from the gel using a sterile 3 mm diameter biopsy punch to avoid contamination of gel samples with environmental proteins. Gel plugs were collected and stored in ultrapure water at -20 °C. Gel plug samples (annotated as 1.4, 2.3, 3.1, 3.2 and 4 (Fig. 4.1 B)) were sent to Plateforme Proteomique Centre de Recherché du CHU de Québec CHUL, Québec, Canada for MS analysis.

4.2.7 Tryptic digestion

Protein digestion and MS analyses were performed by the Proteomics Platform of the CHU de Québec Research Centre (Quebec, Canada). Excised gel pieces were placed in 96-well plates and then washed with water followed by tryptic digestion performed using a liquid handling robot (MultiProbe, Perkin Elmer) according to the manufacturer's specifications. Briefly, proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 126 nM of modified porcine trypsin (Sequencing grade, Promega, Madison, WI) at 37°C for 18 h. Digestion products were extracted using 1% formic acid, 2% acetonitrile followed

by 1% formic acid, 50% acetonitrile. The recovered extracts were pooled, vacuum centrifuge dried and then resuspended into 12 µl of 0.1% formic acid and 5 µl were analyzed by MS.

4.2.8 Mass spectrometry

Peptide samples were injected and separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ESI MS/MS). The experiments were performed with a Dionex UltiMate 3000 nanoRSLC chromatography system (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific,) driving with Orbitrap Fusion Tune Application 2.0 and equipped with a nanoelectrospray ion source. Peptides were trapped at 20 µl/min in loading solvent (2% acetonitrile, 0.05% TFA) on a 5mm x 300 µm C18 pepmap cartridge pre-column (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) during 5 min. Then, the pre-column was switch online with a self-made 50 cm x 75µm internal diameter separation column packed with ReproSil-Pur *C18-AQ* 3-µm resin (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) and the peptides were eluted with a linear gradient from 5-40% solvent B (A: 0,1% formic acid, B: 80% acetonitrile, 0.1% formic acid) in 30 min at 300 nL/min. Mass spectra data were acquired using a data-dependent acquisition mode using Thermo XCalibur software version 3.0.63. Full scan mass spectra (350 to 1800m/z) were acquired in the orbitrap using an AGC target of 4e5, a maximum injection time of 50 ms and a resolution of 120 000. Internal calibration using lock mass on the m/z 445.12003 siloxane ion was used. Each MS scan was followed by the acquisition of fragmentation MS/MS spectra of the most intense ions for a total cycle time of 3 seconds (top speed mode). The selected ions were isolated

using the quadrupole analyzer in a window of 1.6 m/z and fragmented by Higher Energy Collision-induced Dissociation (HCD) with 35% of collision energy. The resulting fragments were detected by the linear ion trap in rapid scan rate with an AGC target of 1e4 and a maximum injection time of 50 ms. Dynamic exclusion of previously fragmented peptides was set for a period of 20 sec and a tolerance of 10 ppm.

4.2.9 Database searching

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). The mascot was set up to search the TAX_Desulfovibrio_CI_194924_20160714 database (unknown version, 104802 entries) assuming digestion with trypsin. The mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Deamidated asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Two missed cleavages were allowed.

4.2.10 Criteria for protein identification

The Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (370) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (371). Proteins that contain similar

peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

4.2.11 Bioinformatics analysis of proteins

Amino acid sequences from peptides identified by mass spectrometry were submitted to the BLAST algorithm (372) to identify corresponding proteins. Prediction of functional domains and motifs was performed using UniProt (https://www.uniprot.org) and Pfam (http://pfam.xfam.org) and proteins are listed in Table 2. To compute physical and chemical parameters of MS/MS detected proteins, protein sequences were submitted to the ExPasy ProtParam tool (https://web.expasy.org/protparam) (373). To predict presence of B-cell epitopes, sequences were submitted to BepiPred-2.0, a web server for sequence-based B-cell epitope prediction (http://tools.iedb.org/bcell/) (374).

4.2.12 Molecular cloning

Bacterial genomic DNA from avirulent *L. intracellularis* N343 was isolated using GenElute^{tm} Bacterial Genomic DNA Kit, (Sigma-Aldrich) following manufacturer's protocol. PCR amplification of open reading frames was performed using Phusion® High-Fidelity PCR kit, (NEB, Ipswich, MA, USA). Primers with cleavage sites for in-frame cloning with the N-terminal His-tag contained within the expression vector pET30a are listed in Table 4.1; these were based on the genomic sequence of *L. intracellularis* (PHE/MN1-00). DNA was gel purified, cut with the appropriate restriction enzymes (either *Bam*HI/*Xho*I or *Nco*I/*Xho*I) and ligated into pET30a using

T4 DNA Ligase (NEB, New England Biolabs). The resulting constructs were transformed into competent Dh5α *E. coli* using standard procedures (375). Plasmid DNA were isolated from the bacteria using the Presto Mini plasmid kit (Genaid, New Taipei City, Taiwan). The cloned sequences and vector insertion were validated by DNA sequencing and restriction digests (data not shown)

4.2.13 Expression and purification of recombinant proteins

Recombinant proteins were expressed in LOBSTR-BL21 (DE3) pRosetta2 *E. coli* after transformation with the plasmids. *E. coli* grown grown to mid-exponential phase (OD = 0.6) in 2xYT medium was induced by the addition of IPTG to 0.5 mM. Incubation was continued for 16 h at 16 °C with shaking at 200 rpm. Bacteria were harvested by centrifugation and suspended in urea lysis buffer (8 M urea, 50 mM $NaHPO_4$, 300 mM NaCl) followed by sonication to lyse bacterial cells. The lysate was centrifuged at 20,000 x g for 15 min to remove insoluble material. Recombinant His-tagged proteins were purified from the supernatants using the His60 Superflow Resin (Clontech, Takara Bio USA, Inc., Mountain View, CA, USA) equilibrated with urea lysis buffer. Proteins were purified in accordance with the manufacturer's protocol and eluted proteins were dialyzed in PBS. Recombinant proteins were subjected to SDS-PAGE (rLI1153 10% SDS gel, rFlagellin LI0710 12 % SDS gel, rLI0649 8% SDS gel, and rLI0169 10% SDS gel), and analyzed by Western blot using pigs sera from animals with clinical symptoms of PHE.

Table 4.1. Bacterial isolates, plasmids and primers used in this study

Isolate	Description	Origin
Avirulent *L intracellularis*	Vaccine strain	Live vaccine
Dh5α	*E. coli* cloning host	
LOBSTR-BL21(DE3) pRosetta2	*E. coli* expression host	Kerafast
Plasmid		
pET30a	IPTG inducible, T7 expression vector, C-terminal 6xHis tag	Novagen
Primers	**Sequence 5' to 3'**	**Restriction site**
Flag-F	GACGGATCCTCTCTTGTCATTAATAACAACCTGATGG	*Bam*HI
Flag-R	GAGCTCGAGTTAGCCAATAAGTTGCTGAGCC	*Xho*I
LI1153-F	GAGGGATCCGCTAATGTTAGTGGAATCCCTGC	*Bam*HI
LI1153-R	GAGCTCGAGTTATTGTATATTATTTTCATCTGGTTGTAGTG	*Xho*I
LI0649-F	TCCCATGGCTGAGGCTGTTGAACACTTTG	*Nco*I
LI0649-R	GGCTCGAGTTAGAATCTATAAGTAGCTCCTACC	*Xho*I
LI0169-F	CGCCATGGACAGTGATGAGGACCTTAGTACAG	*Nco*I
LI0169-R	AGCTCGAGTAGGAATCCACCACTGATCAAG	*Xho*I

4.2.14 Animals and generation of immune serum

All animal experimental procedures were performed in accordance with the Procedures for Ethics Review of Animal Use Protocols and approved by the University Committee on Animal Care and Supply (UCACS), University of Saskatchewan.

Rabbit serum against whole cell *L. intracellularis* was generated as reported in Obradovic et al (376). To obtain hyperimmune serum specific for each recombinant protein of interest, four female New Zealand White rabbits (2-3 kg weight) were kept in isolation units in the Animal Care Unit of VIDO-InterVac. Rabbits were injected via the subcutaneous route with the inoculum consisting of 100 µg of recombinant protein for the first immunization and 50 µg of the same recombinant protein for two booster injections. For all injections, recombinant proteins were dissolved suspended in 500 µl sterile PBS and mixed with 500 µl sterile Incomplete Freund's adjuvant (Sigma-Aldrich) to 1 ml final volume and the vaccines were administered subcutaneously at 4 injection spots with 250 µl of inoculum per site. Each rabbit received just one of the four recombinant proteins on day 0, 20, and 40. Rabbit immune sera were collected via exsanguination following euthanasia of the rabbits (Euthanyl, Bimeda-MTC Animal Health INC., Cambridge ON, Canada) 60 days after the first vaccination. All blood samples were collected and centrifuged (2500 × *g*) then sera were stored at −20 °C until use.

4.2.15 Removal of antibodies against LPS from rabbit immune serum

To remove any LPS-specific antibodies from sera, we incubated 10000 EU/ml LPS from *E. coli* 055:B5 (Sigma-Aldrich) per ml of each rabbit serum for 1 h at room temperature to allow serum anti-LPS antibodies to bound. After one hour of incubation, the endotoxin removing gel (Pierce High-Capacity Endotoxin removing Gel, Thermo Scientific) was used according to the manufacturer's protocol, to remove LPS and LPS-bound antibodies from rabbit sera. The Flow-through fractions before and after elution of LPS were collected and subjected to WB to test the efficacy of the clearing procedure. WB was performed on LPS (Lipopolysaccharide from *E. coli*

055:B5, Sigma) as a control, whole cell *L. intracellularis* extract, and all 4 recombinant proteins. Detection was performed using LPS-cleared rabbit serum in 1:500 dilution as primary antibody and anti-rabbit IR 800 antibody (1µg/ml; Li-COR) as secondary antibody (Figure 4.5 B, C).

4.2.16 Neutralization assay

To determine the effect of recombinant *L. intracellularis* protein-specific sera on the penetration of bacteria into IPEC cells, we performed neutralization assay using CFSE-stained bacteria, as previously described (376). Briefly, CFSE was used to stain avirulent *L. intracellularis* and stained bacteria were incubated with low (500 µg/ml), medium (1000 µg/ml) and high (2000 µg/ml) complement-inactivated LPS-free rabbit hyperimmune serum for 1 h at room temperature. Bacteria bound with serum antibodies were used to infect 10^5 IPEC-1 cells in 24 well plates (Corning) incubated in a tri-gas environment (10% hydrogen, 10% carbon dioxide and 80% nitrogen gas (Praxair Canada Inc., Mississauga, ON, Canada)) in zip lock bags at 37 °C (99). After 4 h incubation, cells were trypsinized then centrifuged at 500 x g for 5 min to remove medium and unbound bacteria. The cells were then suspended in PBS (Gibco Life Technologies) with 2 % FBS (Gibco Life Technologies) and analyzed by flow cytometer. This assay was repeated 4 times independently to obtain biological replicates. Flow cytometric analysis was performed using a BD FACS Calibur™ flow cytometer (BD Biosciences, Mississauga, ON, Canada). CFSE fluorescence was detected in the FL1 channel with gating selected based on uninfected IPEC-1 cells (negative control) and IPEC-1 cells infected with CFSE labeled bacteria (positive control). Thirty thousand events were acquired per sample and flow cytometer results were analyzed in Kaluza software (Beckman-Coulter, Indianapolis, Indiana, USA). The percent inhibition was calculated using the

following formula: Percent inhibition = (1- % of fluorescence of CFSE bacteria incubated with serum / % of fluorescence of CFSE bacteria (control)) x 100.

4.2.17 Statistical analysis:

The Shapiro-Wilk normality test was used to determine whether data follows a Gaussian distribution. One way ordinary ANOVA test was used to compare means of values of percentage of inhibition of each serum. All statistical analyses and graphing were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Differences were considered significant if $p < 0.05$.

4.3 Results and Discussion

4.3.1 Identification of bacterial proteins that interact with IPEC cells as putative antigens

In the present work, we utilized 2-DE coupled with WB analysis and MS/MS to identify *L. intracellularis* proteins that interact with IPEC-1 cell's surface proteins and are recognized by rabbit hyperimmune serum. On the WB, Cy-labeled *L. intracellularis* proteins appear as red spots (Fig 4.1A) whereas the green/yellow spots indicated that these proteins were also bound by rabbit antibodies and are therefore immunogenic. Characteristic accumulation of the abundant albumin protein was observed in the region from 75 kDa to 100 kDa which was also confirmed by MS/MS analysis (data not shown). Despite three washes with serum-free medium, contaminating albumin

was consistently present and attempts to remove albumin from the samples failed. However, despite the presence of albumin, low MW proteins separated well and we were able to visualize both red and green spots indicating Cy-labeled *L. intracellularis* proteins alone and bound by antibodies. Using the WB (Fig 4.1A) as a template, we isolated the corresponding spots in the silver-stained preparative gel (indicated by a red circle in Fig 4.1B). These proteins/spots were excised from the gel and subjected to MS/MS analysis.

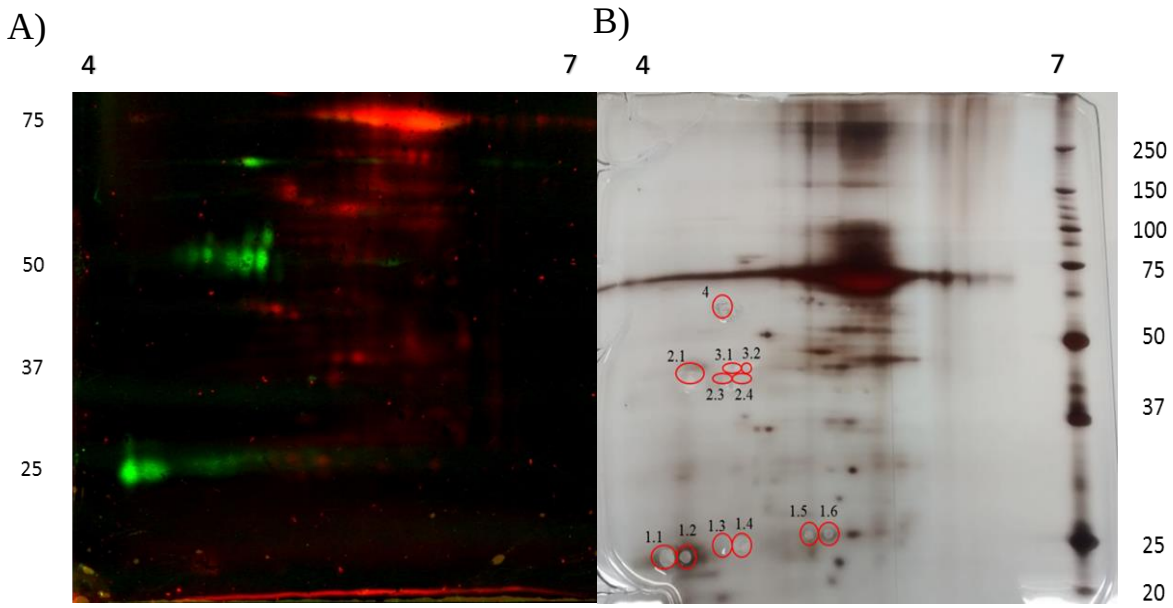

Figure 4.1. Protein separation by 2-Dimensional electrophoresis and selection of spots for mass spectrometry. Proteins were subjected to isoelectric focusing using IPG strip 4-7 (horizontal plane) followed by SDS-PAGE using 10% SDS-PAGE gel (vertical plane). Molecular weight markers are indicated (kD). A) Proteins were transferred to a nitrocellulose membrane and incubated with rabbit hyperimmune serum as primary antibody (1:500) and anti-rabbit IR800 secondary antibody (1:10,000). Proteins visible in IR700 channel are red and indicate all Cye-5 labeled bacterial proteins. The proteins visible in IR800 channel are yellow/green and indicate proteins bound by rabbit antibodies. B) The identical gel was stained with PROTSil-1 silver stain kit. Position and numbering of gel spots are indicated by red circles. Gel plug samples 1.4, 2.3, 3.1, 3.2 and 4 were sent to Plateforme proteomic Centre de recherché du CHU de Québec CHUL, Québec, Canada for Mass Spectrometry analysis.

Bioinformatics analysis of MS/MS detected proteins by UniProt and PFAM revealed 11 unique bacterial proteins identified by MS (Table 4.2). Four of the indicated proteins were

predicted to be expressed and localized to the outer membrane of bacteria and these were selected for further analysis. These proteins were identified as Flagellin (FliC, LI0710), putative outer protein N (LI1153), ABC dipeptide transport system (LI0169), and a previously described autotransporter LatA (LI0649) (103). The selected proteins were analyzed with BepiPred-2.0, a web server analysis tool for sequence-based B-cell epitope prediction (374). All four protein sequences were predicted to have significant presence of B-cell epitopes thus suggesting their potential immunogenicity *in-vivo* (Fig 4.2). Among the 11 proteins detected in our study, proteins Chaperonin GroeL (LI0625) and 5'-nucleotidase/2', 3' cyclic phosphodiesterase (LI1171) were also reported previously using a shotgun proteomic approach which increased our confidence in our approach (104).

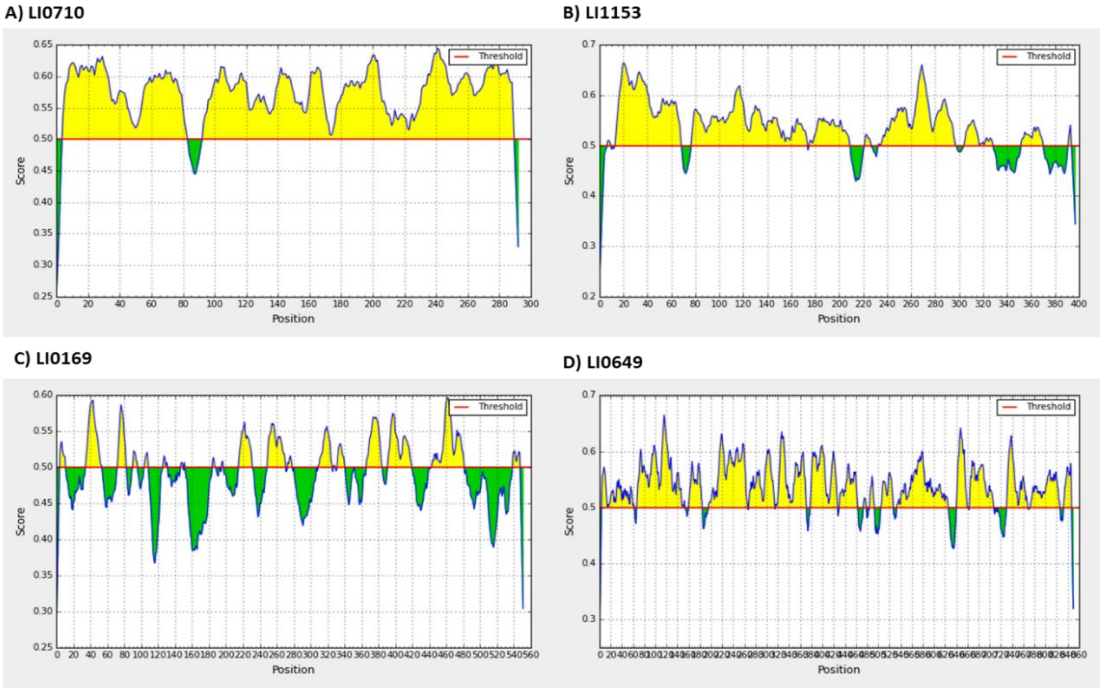

Figure 4.2. B-cell epitope prediction of four *L. intracellularis* proteins. Protein sequences were submitted to BepiPred-2.0 analysis tool to predict B-cell epitops. LI0710 (A), LI1153 (B), LI0169 (C) and LI0649 (D). Yellow peaks represent parts of protein sequence with high score (above the threshold) and green peaks represent parts of protein sequence with low score (bellow the threshold) for predicted presence of B-cell epitope.

Flagellin (LI0710, FliC; NCBI-protein: CAJ54764; UniProt: Q1MQG3, MW 31kDa, PI 5.97 ExPasy) is a subunit protein that polymerizes to form flagella and plays an important role in bacteria locomotion and chemotaxis. Flagellum was observed as a bacterial cell structure of *L. intracellularis* by Lawson and Gebhart (6). Recently, a *L. intracellularis* recombinant protein similar to flagellin (LI0710) (identified as flagellar associated protein, LFliC, LI0570) was shown to be immunogenic and to induce expression of IL-8 in HEK-Blue™-hTLR5 cells in a TLR5-specific manner (105). The flagellin LI0710 and the putative protein LI0570 (NCBI-proteinID: CAJ54624; UniProt: Q1MQV3) share 79.8% amino acid sequence similarity with highly conserved domains although they are coded by distinct regions on the bacterial genome. The gene encoding LI0710 resides between 894,199 and 895,080 nt, whereas LI0570 resides between 701,958 and 702,842 nt of the bacterial chromosome. The flagellin LI0710 has 293 amino acids and consists of a PF00669 (PFAM) domain between amino acids 5 to 141 and a PF00700 (PFAM) domain between amino acids 208-291.

Table 4.2. *L. intracellularis* proteins detected by mass spectrometry

PHE/MN1-00 Locus tag	NCBI PHE/MN1-00 annotation	Spot	Sequence coverage %	# Peptides identified	Probability %	MW (kDa)/PI (ExPasy)
LI0710	Flagellin, FliC	2.3	36	9	100	31/5.97
LI0649	Autotransporter	4	4	4	100	91.86/4.81
LI0169	ABC type dipeptide transport system	4	4	2	100	63.615/6.52
LI1153	Putative outer protein N	3.1	7	2	100	44/4.62
LI0786	DNA polymerase III subunit beta	3.2	13	6	100	43.622/4.70
LI1171	5'nucliotidase/2'3' cyclic phosphodiesterase	4	11	5	100	62.294/5.71
LI0608	Cysteine-tRNA ligase	4	5	2	100	55.492/5.55
LIO726	S-adenosylmethionine synthase	2.3	6	2	100	44.352/5.41
LI0823	Xaa-Pro aminopeptidase	2.3	5	2	100	40.953/5.6
LI0625	60 kDa chaperon, groL	3.2	5	2	100	58.641/5.63
LI0794	ATP-dependant Clp protease proteolytic subunit	1.4	21	4	100	23.465/4.73

Flagellin is a TLR5 agonist that plays an important role in the immune recognition of Gram-negative bacteria. For example immunization of mice by the systemic route with *Yersinia pseudotuberculosis*-derived Flagellin triggered protection in mice orally infected with *Y. pseudotuberculosis*, likely through the activation of TLR5 receptors on intestinal epithelial cells and $CD103^+$ DCs in the *lamina propria* (377). These mice showed increased secretion of flagellin-

specific IgA antibodies in the intestinal mucosa (168) which likely played a primary role in protection. Due to its dual antigen and adjuvant nature, we predict that *L. intracellularis* flagellin LI0710 may be an ideal subunit vaccine candidate antigen.

The LI1153 protein, annotated as a putative outer protein N (NCBI-protein: CAJ55207; UniProt: Q1MP70; MW 44 kDa; PI 4.62, ExPasy) is a part of the T3SS system. The LI1153 protein consists of two prominent domains with important functions during invasion into eukaryotic cells. The HrpJ domain, positioned between 63-222 amino acids, PF07201 (PFAM) and TyeA domain positioned between 299-378 amino acids, PF09059 (PFAM). The HrpJ domain is predicted to be part of the T3SS and related proteins include SsaL and InvE invasion protein from *Salmonella* Typhimurium which are involved in host-pathogen interaction (378) and invasion (379), respectively. A related *E. coli* protein, SepL, plays a crucial role in the infection of enterohemorrhagic *E. coli* and has a potential role in the secretion of EspA, EspD, and EspB (380). The TyeA domain was identified in *Yersinia spp*. and it plays an essential role in controlling the secretion of effector proteins, YopE and Yop H (but not YopM, YopO, and YopP) into eukaryotic cells (381). TyeA is localized at the bacterial surface and together with YopD and YopN contributes to a translocation-control apparatus within the T3SS (381). These secretion regulator proteins have been described as "gate-keepers" in major Gram-negative bacterial species and their deletion leads to decreased secretion of translocon proteins or increased secretion of effector proteins (382). Alberdi et al 2009 determined that one part of *L. intracellularis* T3SS was positioned between 660,438 nt and 661,754 nt in bacterial genome and that the second part of T3SS has positioned approximately 800 kbp distant from their reported region between 1,413,348 nt and 1,414,535 nt (368). We predict that the LI1153 protein, annotated as Putative Outer protein N, corresponds to the predicted second part of the *L. intracellularis* T3SS and has a role in

controlling the secretion of effector proteins into host cells. Further structural and functional studies are needed to elucidate the interactions of LI1153 with T3SS effector proteins of *L. intracellularis* but given our observation of an interaction between this protein and target cells, we believe the protein is likely to play a role in invasion and attachment of the bacteria to small intestine enterocytes.

LI0169, OppA (NCBI-proteinID: CAJ54225; UniProt: Q1MS01; MW 63.5 kDa and PI 6.59, ExPasy) is coded by the gene *oppA* and is predicted to be expressed on the bacterial membrane as part of the ABC transporter complex. In bacteria, the ABC transporter complex plays a central role in the uptake of sugars, amino acids, metals, growth factors, ions and other solutes across the cell membrane (383). The LI0169 protein consists of the transmembrane helical domain (12-34 aa), a periplasmic domain (98-456 aa, PF00496) and an ATP-binding coiled domain (476-496 aa) at the intracellular face of the membrane that together forms a central pore. It transports di- and tripeptides in an ATP-dependent manner (384).

The protein LI0649 (NCBI-protein ID: CAJ54703; UniProt: Q1MQM4; 91.861 kDa, pI 4.81, ; ExPasy) has been identified previously as the autotransporter protein LatA (103). Using an approach similar to ours involving WB analysis coupled with MS, Watson et al, 2011 determined that LatA was recognized by pig hyperimmune serum thus representing a potential target for use in immunodiagnostics. The reoccurrence of this protein in our results, further validates the addition of 2-DE, confirms that LatA is immunogenic as it is bound by rabbit anti-*L. intracellularis* hyperimmune serum and has a role in bacterial-host interactions. The LatA protein had a predicted molecular mass of 91.2kDa (as determined by ExPasy) but the corresponding protein was 60 kDa in our 2-DE SDS-PAGE gel suggesting that cleavage of the protein may have occurred. This

protein is immunogenic and represents a good candidate for making recombinant protein and formulate subunit vaccine.

4.3.2 *In vitro* evaluation of recombinant protein antigenic properties

The bioinformatics analysis of these four proteins predicted to be localized to the bacterial surface and therefore they are potentially accessible to host antibodies. We selected these proteins for expression in *E. coli* and further analysis. Recombinant proteins were expressed into LOBSTR-BL21 (DE3) pRosetta2 *E. coli*. SDS-PAGE analysis and Coomassie staining (Fig 4.3A) confirmed that rLI1153 (44 kDa; Lane 1), rLI710 Flagellin (32 kDa; Lane 2), rLI0649 (92 kDa; Lane 3), rLI0169 (64 kDa; Lane 4) were expressed at their predicted molecular masses. To confirm that these recombinant proteins were immunogenic in pigs, we pooled serum from pigs diagnosed with PE from a *L. intracellularis* endemic farm and used the sera in a WB analysis. All four recombinant proteins were recognized by sera from PE-infected pigs (Fig 4.3B; rLI1153 (44 kDa; Lane 1), rLI0710 FliC (32 kDa; Lane 2), rLI0649 (92 kDa; Lane 3), rLI0169 (64 kDa; Land 4)). This indicates that these recombinant proteins remained immunogenic and demonstrates the relevance of using rabbit serum to detect antigens for causative agent of PPE. The rLI0649 protein was weakly recognized by sera from PE-infected pigs in the WB possibly due to the fact that pig sera were pooled from only 5 infected animals from one farm. Previously studies showed that the pig sera against *L. intracellularis* from different animals have variable intensity of binding to recombinant proteins, rLI0649 (103) or rLscQ (368), possibly due to the differences in immune response against bacteria or infectious doses of bacteria infecting each animal.

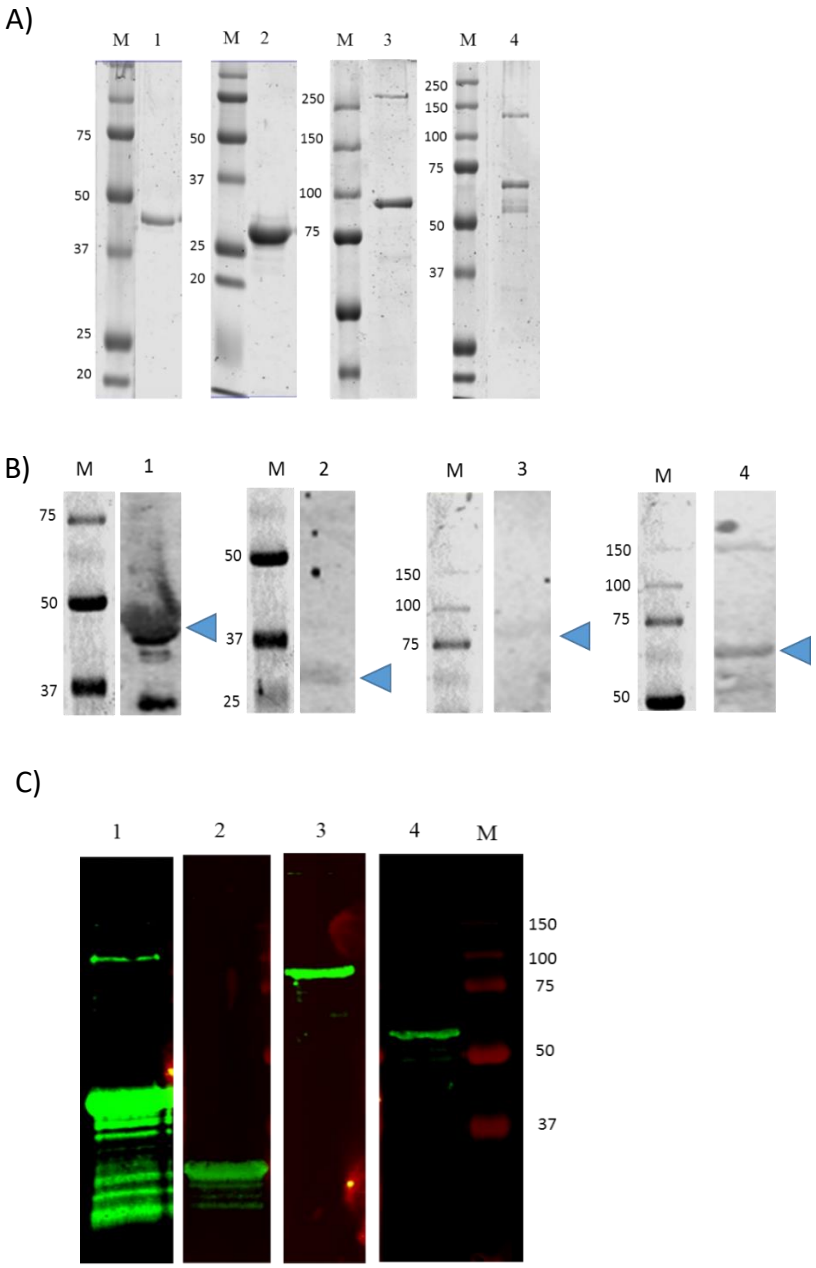

Figure 4.3. Coomassie stained gels with Ni purified recombinant proteins and Western blot analysis. Marker lanes are indicated by M and sizes in kd are indicated. A) Lane 1: rLI1153 44kDa (10 % SDS gel), Lane 2: rLI0710 fliC 32kDa (12 % SDS gel), Lane 3: rLI0649 92kDa (8 % SDS gel), Lane 4: rLI0169 64kDa (10 % SDS gel). B) Recognition of purified recombinant proteins by sera pooled from pigs with clinical symptoms of PHE (1:500). Anti-pig IR 800 (1:10000) was used as the secondary antibody. Arrows indicate predicted MW or recombinant proteins. Lane 1: rLI1153 44kDa, Lane 2: rLI0710 31kDa, Lane 3: LI0649 92kDa, Lane 4:LI0169 64kDa. C) Western Blot analysis of recombinant proteins. For all blots, recombinant proteins were recognized by antigen-specific IgG purified form rabbits immunized with recombinant proteins (1:500) followed by the secondary anti-rabbit IR800 antibody (1:10,000). Rabbit sera were generated against recombinant protein LI1153 44kDa (Lane 1), recombinant protein LI0710 fliC (MW 32kDa) (Lane 2), recombinant protein LI0649 (MW 92kDa) (Lane 3); and recombinant protein LIi0169 (MW 64kDa) (Lane 4).

To test the immunogenic potential of each of the 4 recombinant proteins, we vaccinated rabbits with the recombinant proteins to generate hyperimmune serum specific for each protein target. Hyperimmune serum was then used in WB analysis and visualized with anti-rabbit secondary IR800. Fig 4.3C, lane 1 shows recombinant LI1153 bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI1153). The band at approximately 100 kDa and the smaller bands at 35 kDa are *E. coli* proteins present after Ni-affinity column purification.

Lane 2 shows the recombinant LI0710 Flagellin (32 kDa) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant flagellin). Lane 3 shows the recombinant LI0649 protein (92 kDa) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI0649). Finally, lane 4 shows recombinant LI0169 (64 kDa) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI0169). Serum from unimmunized rabbits did not recognize any of our four recombinant proteins (Fig. 4.4). Our results indicate that the recombinant proteins were recognized by rabbit hyperimmune sera thus indicating their immunogenic properties.

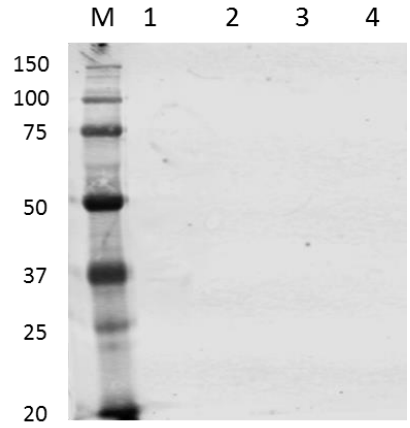

Figure 4.4. Western blot of recombinant proteins probed with precleared negative sera (sera before immunization). Putative protein N LI1153 (Lane 1), rFlagellin LI0710 (Lane 2), rLI0649 (Lane 3) and rLI0169 (Lane 4) were probed with pooled rabbit negative sera (1:500) that had been pre-cleared to remove anti-LPS antibodies. Secondary anti-rabbit IR800 (1:10000) used as negative control. Lane M, molecular weight marker.

4.3.3 *In vitro* evaluation of proteins antigenic properties using flow cytometry

We performed a neutralization assay (methodology optimized in Obradovic et al 2016 (376) to quantify the level of inhibition that antigen-specific antibodies had on preventing penetration of CFSE-labelled avirulent *L. intracellularis* into eukaryotic cells (Fig. 4.6). The following sera were tested at low (500 μg/ml), medium (1000 μg/ml) and high (2000 μg/ml) concentrations: rabbit sera before immunization, rabbit sera against whole avirulent bacteria and rabbit hyperimmune serum specific for rLI0169, rLI0649, rLI0710 FliC, or rLI1153. A MOI of 0.1 of CFSE stained *L. intracellularis* remained constant in all the assays. As our negative control sera, we pooled the sera from rabbits prior to immunization for hyperimmune serum generation. Because others have shown that a negative mouse serum showed 48% to 59% inhibition of *L. intracellularis* invasion, likely due to the presence of anti-LPS antibodies present prior to generation of hyperimmune serum (385), we tested whether negative rabbit serum was capable of binding to LPS. We observed that rabbit negative serum did bind to one band with a molecular mass between 20 and 25 kDa (Fig. 4.5A) which corresponds to the molecular weight of LPS (27). Therefore, we removed anti-LPS antibodies from all sera prior to performing the neutralization assays. WB analysis of sera pre- and post-incubation with LPS to remove LPS binding antibodies is shown in figure 4.5.

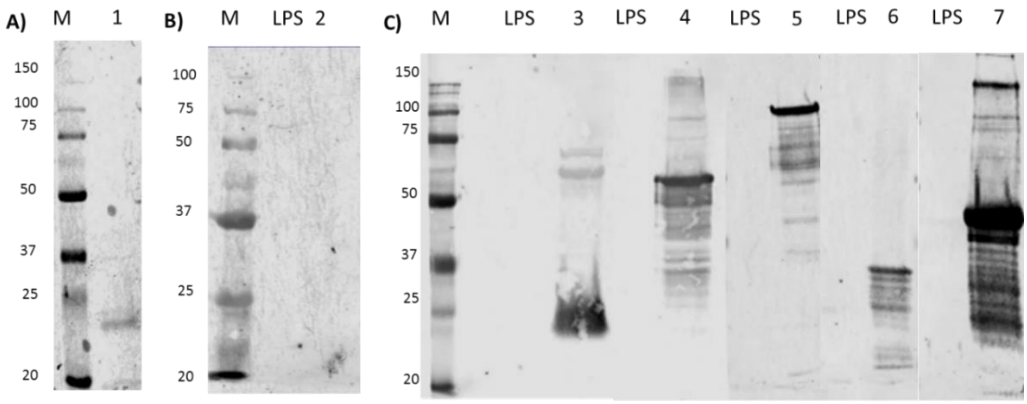

Figure 4.5. Removal of LPS-specific antibodies from sera: Western blot analysis of whole *L. intracellularis* bacteria detected with negative control rabbit sera (sera before immunization with recombinant proteins), protein marker M, whole *L. intracellularis* bacteria (lane 1), (A); WB with negative sera after specific antibodies removal, LPS and whole *L. intracellularis* bacteria (lane 2) (B); WB with sera generated against whole avirulent *L. intracellularis* after specific antibodies removal, LPS, *L. intracellularis* (lane 3), WB with sera generated against recombinant proteins: LPS, rLI0169 (lane 4), rLI0649 (lane 5), rLI0710/fliC (lane 6) and rLI1153 lane 7 (C). Rabbit sera were used in 1:500 to detect proteins within *L. intracellularis*, primary antibody targets were detected with secondary anti-rabbit IgG 1:10000 IR 800.

The gate in flow cytometry analysis was based on percentages of fluorescence detected in FL-1 channel (Gate B from representative biological replicate shown in Figure 4.6) for IPEC-1 cells alone (Fig 4.6A) and IPEC-1 cells infected with CFSE-stained *L. intracellularis* (Fig 4.6.B). As expected, IPEC-1 cells alone had negligible positive fluorescence events (mean value of 0.20% fluorescence in FL1 channel; Fig 4.6A) and IPEC-1 cells infected with CFSE-stained *L. intracellularis* showed a mean value of 8.86% fluorescence in FL1 channel, 4 hours post-infection (Fig 4.6B). The percentage of positive events in FL1 channel when CFSE-stained *L. intracellularis* was incubated with 2000 μg/ml of serum from a rabbit immunized with whole bacteria was reduced to 2.29% (Fig 4.6C). Figure 4.7 shows the percentage of inhibition of *L. intracellularis* invasion of IPEC-1 cells in the presence of serum antibodies. We observed that pre-incubation of CFSE-*L. intracellularis* with low (500 μg/ml), medium (1000 μg/ml) and high (2000 μg/ml) concentrations of negative control sera showed 46.7% (±7.9), 58.9% (±8), 65.7% (±5.7) inhibition. This inhibition by negative control serum is not unexpected as others have shown that rabbit

polyclonal sera prepared against *E. coli* and other negative control serum obtained prior to generation of *L. intracellularis* hyperimmune sera inhibited *L. intracellularis* penetration of cultured rat enterocytes (IEC-18) (385).

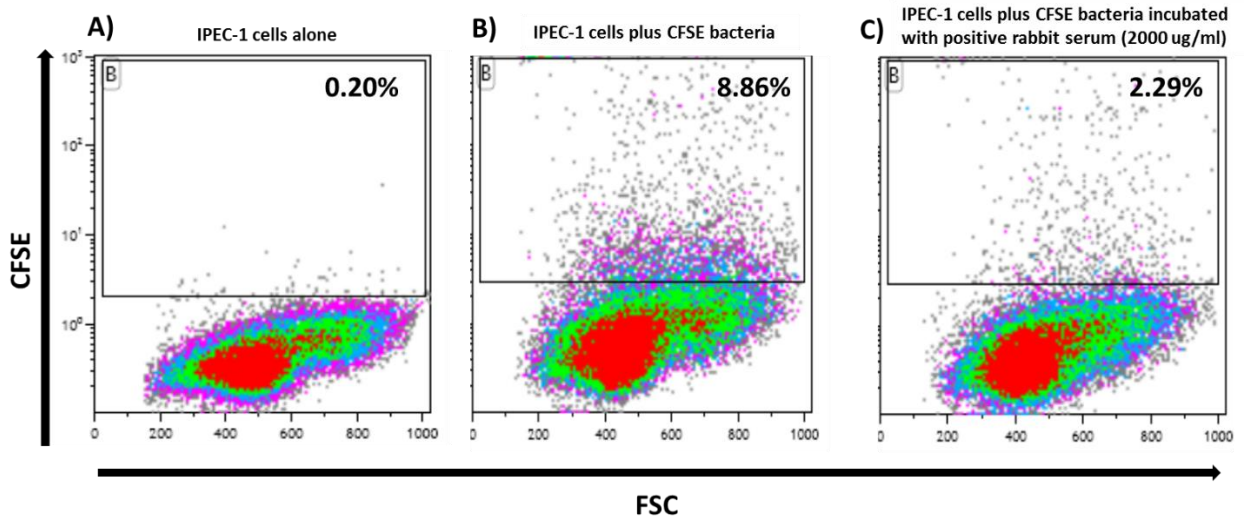

Figure 4.6. Representative dot blots and gating for flow cytometry analysis: A) gates on live IPEC-1 cells forward scatter/CFSE plot, 0.22% of fluorescent events, B) IPEC-1 cells infected with CFSE- labeled *L. intracellularis* MOI 0.1, 8.86% of fluorescent events, C) IPEC-1 cells infected with CFSE- labeled *L. intracellularis* MOI 0.1 incubated with 2000 μg/ml of positive serum from rabbits vaccinated against whole bacteria, 2.29% of fluorescent events (C). Plots represent one of four biological replicates for all sera purified from LPS antibodies to obtain mean values and calculate the level of inhibition. Thirty thousand events were acquired for each sample.

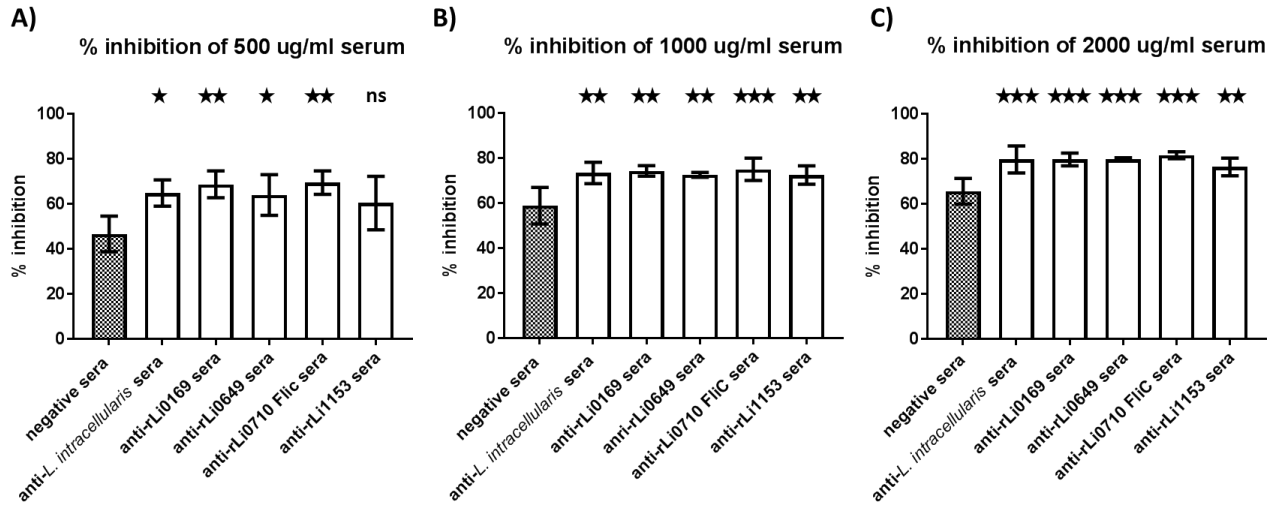

Figure 4.7. Inhibitory effect of rabbit serums on CFSE labeled avirulent *L. intracellularis* penetration in IPEC-1 cells: negative control sera (sera obtained prior to immunization and pooled), anti-L. intracellularis sera (serum from rabbits immunized with whole avirulent bacteria), sera from rabbits immunized with recombinant proteins: anti-rLI0169, anti-rLI0649, anti-rLI0710 FliC, anti-rLI1153; serum concentrations used in assay 500 µg/ml (A); 1000 µg/ml (B) and 2000 µg/ml (C). All sera were cleared from antibodies against LPS. Percent inhibition = (1- % of fluorescence of CFSE bacteria incubated with serum / % of fluorescence of CFSE bacteria (control)) x 100. Data presented for 4 biological replicates. The bar shows the standard deviation of mean value of 4 biological replicates. ((***) $p<0.001$, (**) $p<0.01$ and (*) $p<0.05$, (ns) not significant.

We used rabbit hyperimmune generated against whole *L. intracellularis* as our positive control serum and results indicated that CFSE-labeled *L. intracellularis* incubated with low (500 µg/ml), medium (1000 µg/ml) and high (2000 µg/ml) serum resulted in 64.8% (±5.7), 73.4% (±4.7) and 79.88% (±5.9) inhibition of infection (Figure 4.7 A-C). Relative to the negative control sera, a positive control serum inhibited significantly more cellular adhesion/ penetration at low ($p<0.05$; Fig 4.7A), medium ($p<0.01$; Fig 4.5B) and high ($p<0.001$; Fig 4.7C) sera concentrations indicating that anti-*L. intracellularis* antibodies were neutralizing. To discern whether antibodies specific for rLI0169, rLI0649, rLI0710 and rLI153 blocked bacterial adherence/penetration into IPEC-1 cells, we pre-incubated the CFSE-stained *L. intracellularis* with 500 µg/ml (Fig 4.7A), 1000 µg/ml (Fig

4.7B) and 2000 μg/ml (Fig 4.7C) hyperimmune sera specific for each recombinant protein. At the lowest concertation of hyperimmune sera (Fig 4.7A), anti-rLI0169 showed 68.7% ±5.9 inhibition, anti-rLI0649 showed 64% ±9.0 inhibition, anti-rLI0710/FliC showed 69.5% ±5.2 inhibition, and anti-rLI1153 showed 60.4% ±11.8 inhibition. With the exception of anti-rLI1153, all 3 hyperimmune sera showed significantly higher percent inhibition relative to the negative control serum ($p<0.01$, $p<0.05$, $p<0.01$, respectively). When the medium and high concentration of each antisera was used, statistically significant reduction of bacteria entrance into IPEC-1 cells was achieved relative to the corresponding dose of control serum: anti-rLI0169 ($p<0.01$ Fig 4.7B, $p<0,001$ Fig 4.7C), anti-rLI0649 ($p<0.01$ Fig 4.7B, $p<0.001$ Fig 4.7C), anti-rLI0710/FliC ($p<0.01$ Fig 4.7B; $p<0.001$ Fig 4.7C), and anti-rLI1153 ($p<0.01$ Fig 4.7B; $p<0.01$ Fig 4.7C). We can conclude that sera antibodies specific for each recombinant proteins showed the comparable inhibitory effect to that observed with the positive rabbit serum against whole bacteria and that the inhibitory effect of all sera increased with increased serum concentration. The results from recombinant serum neutralization assay suggest that use of recombinant proteins as antigens in subunit vaccine formulation may generate neutralizing antibodies capable of inhibiting *L. intracellularis* penetration and infection.

Because *L. intracellularis* is an obligate intracellular bacteria, the cellular immune response is assumed to play the major role in protection against virulent bacteria (284, 285), however, the humoral immune response may also play an important role in protecting against *L. intracellularis* infection. IgG antibodies against intracellular bacteria could bridge humoral and cellular immunity by targeting of intracellular pathogens to lysosomes through antibody-FcR-mediated stimulation of the host cells (386), protection against intracellular bacteria by an Fc

receptor-mediated lysosomal targeting (387) and modulation of cytokine secretion (388). Also, IgA antibodies play an important role in protection against enteric pathogens. Accumulation of IgA bound to *L. intracellularis* inside enterocytes and *lamina propria* was reported previously (365) and *L. intracellularis* specific IgA were detected in intestinal lavage of pigs 3 weeks after experimental infection (285). Results from a vaccine trial where animals were vaccinated orally and challenged with virulent *L. intracellularis* suggested that protection was associated with mucosal cytokine and specific IgG and IgA responses and that systemic antibody response were boosted following challenge (283). In this study, we demonstrate that sera generated against recombinant proteins inhibited *L. intracellularis* invasion of IPEC-1 cells *in vitro*. Although this study was designed to identify proteins capable of generating a humoral response, it does not preclude the capacity of these proteins to elicit a cellular immune response and thus further investigation in this area is warranted.

A subunit vaccine comprised of Flagellin alone or coupled with these 3 other antigens may induce a specific protective immune response against *L. intracellularis* in intestinal mucosa of pigs and should be investigated further.

In conclusion, we have used functional 2-DE and WB analysis coupled with MS to identify potential antigens for an *L. intracellularis* subunit vaccine in pigs. Because the commercially available *L. intracellularis* vaccines are dependent on the growth of this obligate intracellular pathogen, production may be limited and time-consuming. A subunit vaccine has the advantage that recombinant antigen production can be performed in *E. coli* which is a safe, rapid and a cheap alternative to a vaccine that requires growing, attenuating and inactivating *L. intracellularis*. Flagellin has adjuvant and antigenic properties that induce a specific immune response in intestinal mucosa of mice thus subsequent vaccine with all four recombinant proteins may not require

additional adjuvants to provide protection against the intestinal pathogen. Extensive *in vivo* studies will need to be performed to ensure that such a quadrivalent subunit vaccine is effective in generating protective long-term immunity. However, an effective subunit vaccine against *L. intracellularis* may present the industry with an effective and economically attractive vaccine to use in high-security pig barns.

Student contribution: M.O. designed and performed all the experiments here-in, and wrote the manuscript. Mass Spectrometry Analysis was performed at Plateforme protéomique Centre de recherche du CHU de Québec CHUL, Québec, Canada. I would like to thank Dr. Sylvie Bourassa for assistance with MS/MS.

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 General discussion

L. intracellularis has been detected in swine herds all over the world with high prevalence among intensive production systems (5). Clinical symptoms range from weight loss and diarrhea to profuse bleeding and mortality, which imposes a significant economic burden for the swine industry (6, 48). *L. intracellularis* has been detected in many mammals and ratite birds but has emerged as important pathogen in horses in recent years (2). Rodents shed *L. intracellularis* in their feces and present a possibly important route of transmission for pigs and horses. Treatment and prevention of PPE rely on applying hygiene measures, antibiotic treatments and vaccination. Currently, two vaccines are registered for prevention of PPE in pigs. Enterisol® is formulated with a live avirulent strain of *Lawsonia* attenuated by extensive passaging in cell culture, and the Porcilis® is formulated with a killed bacterin. Both vaccines rely on bacterial growth in cell culture, which has its drawbacks and limitations. Further, immune protection achieved from these vaccines in the field has not always been obtained, thus indicating the need for new effective vaccine. We set out to explore the possibility of the development of a subunit vaccine formulated from recombinant proteins that induces neutralizing antibodies in the host immune system. This type of vaccine formulation may be a welcomed vaccine alternative for the industry.

The obligate intracellular nature makes it a challenge to culture *L. intracellularis* which has been an obstacle for studying these bacteria in laboratory and field conditions. Due to limited tools and techniques currently available, it remains challenging to detect, track and quantify these obligate intracellular bacteria. Thus developing new high through put assays based on accurate quantitative methods, such as flow cytometry and qPCR, offer important tool to explore *L.*

intracellularis pathogenesis. The bacterial components and mechanism that facilitate attachment of bacteria to enterocytes has not yet been fully determined (3), and the bacterial proteins that facilitate invasion of intestinal epithelial cells are perspective immunogens. The goal of this thesis was to identify and use these immunogenic proteins to develop recombinant protein vaccine which could present new options for prevention of PPE that is a safe, effective and attractive vaccine option for both the pig industry and pharmaceutical companies. Also, this research details the development of new quantitative methodology to track intracellular bacterial infection and evaluation of inhibitory effects of immune sera on bacterial penetration as the means to identify neutralizing antibody targets for use in vaccine development.

The first part of the research outlined in this thesis focused on development of functional assays as a useful tool in evaluating intracellular bacterial penetration and the role of serum antibodies in inhibiting that penetration and infection or eukaryotic cells. Previously, others showed that CFSE may be useful to label extracellular bacteria to detect the presence and activity of environmental and food-borne microorganism (352, 353, 354, 355, 356, 357, 358). We adapted this approach to label *L. intracellularis* and to use flow cytometry to quantify invasion or cellular association of *L. intracellularis* within its host cell. CFSE dye has staining properties that allow tracking of bacteria with no negative effects on bacterial adhesion, viability, and metabolic activity which helps us compensate for the restriction in use of standard molecular and microbiological techniques to analyze *L. intracellularis*-eukaryotic cell interactions. In light of *L. intracellularis* research, CFSE staining has additional advantages due to the obligate intracellular nature of bacteria and obvious restriction in use of standard molecular and microbiological techniques to analyze the host-pathogen interactions. Using this approach, we confirmed that the CFSE dye is retained in the bacteria and does not leak to the surrounding media or eukaryotic cytoplasm. Using

flow cytometry to detect. CFSE-stained *L. intracellularis* showed improved accuracy over standard *L. intracellularis* enumeration methods, which often show significant discrepancy between different laboratories and require use of specific monoclonal antibodies.

CFSE-stained *L .intracellularis* were used to adhere to or infect McCoy cells. To confirm bacterial invasion, qPCR analysis was applied. qPCR analysis confirmed that the bacteria invaded the host cell and were able to grow in course of 5 days which was represented as increase of bacterial DNA compared to the non-infected cell control and to the day 1 and day 3 of infection. In addition fluorescence-activated cell sorting (FACS) was used to sort infected from non-infected cells for further analysis and confocal microscopy revealed CFSE stained bacteria in apical part of eukaryotic cell cytoplasm surrounding the nucleus. Next, experiments were executed to evaluate whether this approach was compatible with functional analysis. Rabbit hyperimmune serum generated against whole *L. intracellularis* was evaluated for its capability to inhibit entrance of bacteria inside McCoy cells. Interestingly instead of blocking bacterial invasion of the cells, rabbit serum enhanced the entrance of *L. intracellularis* into McCoy cells which was detected both with flow cytometry and qPCR analysis. When the same assay was applied using IPEC-1 cells, porcine intestinal epithelial cell line, rabbit serum against *L. intracellularis* was able to significantly reduce bacterial penetration into IPEC-1 cells (Fig 4.6 and 4.7). The increase of bacterial invasion of McCoy cells in presence of positive rabbit serum is not receptor-mediated and may be due to the fibroblast nature of McCoy cells that can engulf antibody-coated bacteria. Although this cell line was found to be optimal for *L. intracellularis* propagation both in the laboratory and in industrial settings, our results indicate that it is not adequate for use in functional assay due to the different mechanism of bacterial invasion. Further, others showed differential expression of cell cycle and cell differentiation genes of McCoy cells infected with *L. intracellularis* but no proliferative

changes or cell cycle alterations were observed microscopically (83). Marked difference between *in vivo* infection of enterocytes and *in vitro* infection of McCoy cells may indicate that functional assays could not be faithfully reproduced in McCoy cells although they did help prove the validity of CFSE bacterial staining to quantify bacterial invasion of eukaryotic cells. This approach can be adapted for use with facultative intracellular pathogens such as *Neisseria gonorrhea* infection of ME-180 cells (389) and are amenable to be used with *L. intracellularis* infection of for a functional analysis in IPEC-1 cells which are more suitable *in vitro* model for *L. intracellularis* infection.

The obligate intracellular nature of *L. intracellularis* has imposed difficulties in exploring the bacterial components involved in attachment and molecular mechanisms during infection. Immunogenic proteins that play a role in adherence or invasion are not completely characterized and functionally determined. Others have shown that proteins which comprise the Type III secretion system (T3SS) autotransporter proteins (LatA), LI0841, and LI0902 were characterized and shown to have immunogenic properties (368, 103, 104). In the second part of this thesis, the separation power of 2-DE coupled with WB and MS was utilized to detect other uncharacterized bacterial proteins that are localized to the surface localization, that play a role in infection and are immunogens. Using these immunoproteomic techniques, we identified antigens that could be produced as recombinant proteins and utilized in the formulation of effective subunit vaccine. We applied immunoproteomics which yielded 11 unique *L. intracellularis* proteins (Table 4.2). Bioinformatics analysis indicated that four proteins were expressed on the surface of bacteria and have role in pathogenesis. These proteins were identified as flagellin (FliC, LI0710), Putative outer protein N (LI1153), ABC dipeptide transport system (LI0169) and autotransporter LatA (LI0649). Among the 11 proteins detected in this study, proteins chaperonin GroeL (LI0625) and 5'-nucleotidase/2', 3' cyclic phosphodiesterase (LI1171) were also reported previously using a shot-

gun proteomic approach (104). Due to their predicted surface localization, possible roles in pathogenesis and adequate biochemical properties. The flagellin LI0710, LI1153, LI0169 and LI0649 proteins were selected to be cloned, expressed and purified as recombinant proteins. All four recombinant proteins were recognized by sera from PE-infected pigs which indicates that these recombinant proteins remained immunogenic (4.3 B). That being said, we have to acknowledge the limitation of using pooled sera from animals during active infection with *L. intracellularis*. Sera from convalescent animals could have an additional pool of protective antibodies with increased neutralizing or ADCC characteristics. Future experiments should be conducted to compare the capabilities of the sera from actively infected and convalescent animals to recognize *L. intracellularis* proteins and to test the neutralizing characteristics of serum antibodies on bacterial penetration, *in vitro*.

Interestingly, rLI0169 (OppA) was recognized by the pig sera from field cases of PE, indicating that although it has a periplasmic region, this protein is expressed during *L. intracellularis* infection and it is targeted by specific antibodies. OppA is conserved among many bacteria species and these results correspond to reported antigenicity and surface exposure of OppA in *Streptococcus suis* serotype 2 (390). Beyond transporting oligopeptides, Opp transporters are known to have important roles in pathogenesis of different bacterial species (391). They are important factors in adhesion to the host cell in *Corynebacterium pseudotuberculosis* infections (392), survival of *Listeria monocytogenes* inside macrophages (393), and they are expressed during bacteraemia in mouse model of *Staphylococcus aureus* infection (394). To evaluate their potential to induce immune response, each recombinant protein was used to vaccinate rabbits and acquire immune serum. Sera against each recombinant protein was able to recognize corresponding recombinant protein thus indicating their ability to induce immune humoral immune

response when administered as part of an i.m vaccine (4.3 C). Finally, rabbit sera specific for each recombinant proteins succeeded in blocking penetration of *L. intracellularis* into IPEC-1 cells indicating that they are potentially neutralization antigens.

Despite two commercial vaccine based on attenuated live or inactivated whole bacteria currently available to vaccinate pig herds against *L. intracellularis,* there is still room for progress in development of vaccines based on different principles. Subunit vaccines with specific bacterial antigens could be an acceptable alternative vaccine for both pig producers and animal health companies because they trigger immunity to select bacterial antigens without the risk of reversion to virulence. They are also comparatively cheap to produce because unlike inactivated and killed vaccines, a subunit *L. intracellularis* vaccines does not require the bacteria to be propagated in eukaryotic cells which is costly. Most importantly, the commercially available vaccines do not allow one to differentiate between infected and vaccinated animals (DIVA). DIVA vaccines, also known as marker vaccines, have been developed to prevent important swine infectious diseases, like Aujeszky's disease, *Actinobacillus pleuropneumoniae* and Classical Swine fever (395, 396, 397). These vaccines were formulated as live-modified vaccines that lack one or more antigens that are present in wild virulent type thus providing the ability to distinguish between vaccinated and infected animals based on immunological testing. The obligate intracellular nature of *L. intracellularis* limits the use of molecular techniques to genetically modify this organism to express or lack expression of distinctive proteins. The solution to this problem could be development of a DIVA subunit vaccine against *L. intracellularis* based on use of select immunogenic bacterial proteins. For example, one could test for immune response to vaccine subunit antigens as well as an immunogenic protein present on *L. intracellularis* that is not included in the vaccine. Vaccinated animals and infected animals would all have antibodies against

the vaccine antigens but only animals that were infected with *L. intracellularis* would have titres for the immunogenic protein absent from the vaccine. The development of DIVA vaccines have important benefits for pig producers and large animal veterinarians, as they can be used to limit the number of animals culled during an outbreak. Further, the pig industry has ever growing economic pressure and profits are impacted by international trade competition and emergence of new infectious diseases, such as PEDV or African swine fever (398, 399) . Science and technology to develop a subunit DIVA vaccine against *L. intracellularis* can help provide a safe and effective yet affordable vaccine that does not negatively impact trade. Because infected animals can shed *L. intracellularis* for up to 12 weeks (48), pigs testing positive for serum antibodies against *L. intracellularis* may be prevented for entering a barn for fear of transmission of disease. *L. intracellularis* DIVA vaccines could impact sale of replacement stock or movement of animals to different barns. However, if a DIVA vaccine allows the purchasing barn to have confidence that the animals were not infected but were vaccinated, then these animals can be purchased knowing that they pose no risk of transmission of disease to unvaccinated new herd mates. DIVA vaccination would help large animal veterinarians to monitor health status of the herd more accurately and to advise rational use of antibiotics. From the public health perspective, application of DIVA vaccines against *L. intracellularis* is important preventive measure due to possible reduced and more targeted administration of antibiotics to only infected animals and not the whole herd. With the emergence of antimicrobial resistant pathogens and expanding global trade, there are increased regulatory pressures to limit the usage of antibiotics in livestock industry. DIVA vaccines against *L. intracellularis* provide possible effective solution to pig producers for controlling PE and at the same time acceptable preventive measure from the public health

perspective. Although challenges in controlling *L. intracellularis* remain, we can expect new advancements in vaccine development in near future with benefits to all shareholders in industry.

In summary, this study has been able to develop new methodology as a tool to investigate host-intracellular bacteria interactions *in vitro* and identified important *L. intracellularis* proteins involved in infection. Functional assay established that antibodies specific for each recombinant proteins neutralized bacterial invasion, *in vitro*. In future work, *in vivo* experiments should be conducted to analyze immune response and evaluate potential protection against pathogenic challenge of vaccine formulated from these four proteins. This Ph.D. thesis laid the foundation for future experiments and development of safe, effective, protective and economical vaccine against *L. intracellularis*.

5.2 General conclusions

In conclusion, this work developed *in vitro* methodology as a selection process to identify and *L. intracellularis* proteins as suitable subunit vaccine candidates. Functional assays based on CFSE-staining of bacteria and application of flow cytometry were successfully developed to quantify infection of different cell lines and evaluation of effect of specific serum antibodies against *L. intracellularis* on bacterial penetration into eukaryotic cells. Using 2DE and WB analysis coupled with MS, eleven *L. intracellularis* proteins were identified from which four showed potential to be suitable recombinant antigens for formulation of *L. intracellularis* subunit vaccine in pigs. *In vitro* neutralization assay based on flow cytometry indicated that rabbit hyperimmune sera generated against the vaccine strain of *L. intracellularis* and rabbit sera specific for each recombinant protein showed an inhibitory effect on the attachment and penetration of live,

avirulent *L. intracellularis* into eukaryotic cells. Results from this *in vitro* study demonstrated a means to efficiently and economically select potential vaccine antigens prior to *in vivo* studies thus enabling us to narrow down the number of antigens tested in future live vaccine trials. In future, *in vivo* studies will need to be conducted to evaluate that such a quadrivalent subunit vaccine is effective in generating protective long term immunity which will benefit the pig industry.

APPENDIX

A.1 Isolation of pathogenic *L. intracellularis* from infected gut tissue from two PHE field cases in Canada

A.1.1 Introduction

In this chapter, we present data on the procedure to isolate and propagate pathogenic *L. intracellularis* from the intestine of two confirmed cases of PHE. One gut sample originated from gilt from a pig farm in Ontario (received in 2014) and one from a gilt previously vaccinated with Enterisol® vaccine from a farm in southern Saskatchewan (received in January 2018). Both cases were confirmed PHE disease with a typical hemorrhagic form of PE with intestinal lumen filled with blood. Both animals succumbed to the disease and intestinal samples were collected and sent to our lab. Isolation and *in vitro* growth of *L. intracellularis* in McCoy cells from infected intestine was performed according to (4). To monitor infection and passaging of bacteria in McCoy cells, we used specific anti-*Lawsonia* antibody staining detected with flow cytometry. Standard IFAT to quantify infection depends on manual counting of serial dilutions of infected McCoy cells or free bacteria and can be subjective, with great differences between laboratories and even personal among the same lab. As an alternative approach, we used flow cytometry and qPCR to monitor the infection of McCoy cells, *in vitro*. In addition, intestinal homogenate prepared from two cases of PHE were successfully used to infect pigs indicating that the bacteria were infectious.

A.1.2 Materials and methods

A.1.2.1 Pathogenic *Lawsonia intracellularis* isolation from infected gut tissue

Preparation of gut homogenate and isolation of *L. intracellularis* from infected porcine intestines were performed according to (4) and explained in detail in Section 1.2.4.

New *L. intracellularis* isolate stocks were used to infect McCoy cells to start *in vitro* growth. Monitoring of bacteria passaging was done utilizing flow cytometry and presence of bacterial DNA confirmed with qPCR analysis.

A.1.2.2 Cell culture conditions and infection of McCoy cells with *L. intracellularis*

McCoy were grown as reported in (4, (376). For the purpose of propagating *L. intracellularis*, McCoy cells were seeded at a concentration of $0.2x\ 10^6$ per T-25 flask. Medium for cell infection with *L. intracellularis* was Dulbecco's Modified Eagle Medium (DMEM) with 7 % FBS with the addition of vancomycin (100 μg/ml) and amphotericin B (2.0 μg/ml) (4). Frozen aliquots of the gut filtrate and avirulent bacteria were thawed in a water bath on 37°C and diluted in DMEM-7% FBS at ratios 1:10 and 1:20 and added to a 30% confluent monolayer of McCoy cells. The flasks with infected McCoy cells were placed at 37°C in Zip-lock bags filled with a mixture of gases 10% hydrogen, 10% CO_2 and balanced with nitrogen (99). After 3 h, gentamicin (50 μg/ml) was added to the infected cultures.

A.1.2.3 Passage of infection

Cell lysis was acheived by exposing infected McCoy cells to 0.2% KCl for 5 min and then 0.1 % KCl for 20-30 min at 37 ºC. Hypotonic solution was evacuated and 10 ml of DMEM-7% FBS per T-75 or 3 ml per T-25 flask was added, the cells were scraped with a cell scraper and removed to a 50 ml tube. Cells were lysed by passing the suspension repeatedly more than 6 times through a $1^{1/2}$ inch 20-gauge needle in 10 ml or 5 ml syringes. To remove nuclei and cell debris, suspension was centrifuged 150 x g for 5 min. The supernatant was removed and cells collected by centrifugation at 6000xg for 10 min. The pellet was suspended in SPG with 0% FBS and stored at -80 ºC. For passaging, 1:10 of the total volume from one T-25 flask was used to infect next McCoy cells that were growth to 30% confluency.

A.1.2.4 Flow cytometry analysis.

Infected McCoy cells in T-25 flasks were collected for flow cytometry analysis seven days after infection, on the same day when lysing and passaging was done to new T-25 flasks. Controls included non-infected cells, non-infected cells stained with primary and secondary antibody, and non-infected cells stained with isotype control antibodies. The McCoy cells infected with the vaccine strain of *L. intracellularis* served as the positive control. Infected McCoy cells were collected by trypsinization using 5% Trypsin then washed with PBS + 10% FBS, centrifuged at 500 x g for 10 min and this process was repeated twice. Cells were transferred to 96 well plate (Nunclon round bottom wells) and treated with 70% methanol for 5 min at room temperature for cell permeabilization followed by washing with washing buffer (PBS, 0.1% sodium azide, 0.5% BSA). Cells were then incubated for 1 h at room temperature with commercial primary antibody,

mouse anti-*Lawsonia* IgG2b specific antibody (concentration 1:200), 10 µl per well (BIOX, Belgium) or mouse isotype (Chrompure Mouse IgG, Jackson Immuno Research Laboratories, PA, USA). After 1 h of incubation, cells were washed twice with washing buffer (675 x g for 10min) followed by incubation with secondary APC labeled goat anti-mouse IgG2b antibody (Southern Biotech) for 30 min at room temperature in the dark. After washing twice in washing buffer, the cells were resuspended in PBS + 2% FBS and analyzed with a flow cytometer (26). Flow cytometric analysis was performed using a BD FACS Calibur^{tm} flow cytometer (BD Biosciences). We detected fluorescence in the FL4 channel, with gating being selected based on uninfected McCoy cells as our negative control cells. Flow cytometer results were analyzed in Kaluza software (Beckman-Coulter).

A.1.2.5 qPCR analysis to detect *L. intracellularis* specific DNA

McCoy cells were seeded into a T-25 flask with a density of 0.2 x 10^6 per flask. Bacteria lysate from passages was then introduced to the wells and flask with growth medium for bacteria as described before (4). Cells were trypsinized and suspended in DMEM complete media containing 10% FBS to arrest the trypsin reaction. Cells were pelleted by centrifugation at 500 x g for 10 minutes. The pellet was suspended in NaOH (25 mM NaOH, 0.2 mM EDTA) and heated for 95 ^{o}C for 1 h to rupture the cells and release the DNA from the nucleus. HCl (40 mM Tris-HCl, pH 5.5) was added to neutralize the solution. The two types of primers were used, one specific for LI0754 (*L. intracellularis* PHE/MN1-00: LI0754 gene) for qPCR reaction with 50x Kapa Syber Green Fast Rox High kit from Kapa Biosystems, and other 16 S primers (CCGGCTTTGGGTAAAACCA) for qPCR reaction with TaqMan probe (TAMRA TaqMan

probe, AB). Results were analyzed by Step One Real-Time PCR System (Applied Biosystems, by Life Technologies), Excel (Microsoft) and Prism (GraphPad) software.

A.1.2.6 Infection of piglets with isolated pathogenic *L. intracellularis*.

Two separate experiments were conducted to test the infectious potential of intestinal homogenate originated from infected gilts. The first experiment was performed on sixty-day old piglets with intestinal homogenate from the first case of PHE received in 2014. The second experiment was performed on thirty-day old piglets with intestinal homogenate received in 2018. Piglets were infected with intestinal homogenate with 10^8 to 10^9 bacteria orally. After 22 days, piglets were euthanized and blood, fecal, and intestinal samples were collected. For the first trial, intestinal samples were processed in two ways. One part was frozen in liquid nitrogen and ground for PCR analysis and in the second part, intestinal tissue was formalin-fixed, paraffin embedded and mounted on slides for IHC. Anti-*Lawsonia* specific antibody was used as primary and anti-mouse IgG2b conjugated with FITC was the secondary antibody for IHC diagnostic of pathogenic bacteria in pig's small intestine epithelial cells. DAPI was used to stain eukaryotic cell nuclei. Fluorescent microscopy was performed using a fluorescent microscope (Zeiss, Axiovert 200M) and images were taken with Zeiss Axiocam HRC.

For the second trial, intestinal mucosa was scraped and filtered according to standard isolation protocol (4) and *L. intracellularis* isolate was used to infect McCoy cells and propagate *in vitro. In vitro* growth and propagation was monitored using flow cytometry and qPCR as explained above.

A.1.3 Results and discussion

A.1.3.1 Clinical analysis and IHC from Experiment 1

Clinical signs characteristic for PE were not observed in infected animals. There was no signs of watery diarrhea or blood in the feces. Weight of the animals was not significantly altered. Infected animals had normal body temperature and preserved appetite.

Results from our IHC from experiment 1 showed that pathogenic *L. intracellularis* was detected in intestinal samples using anti-*Lawsonia* primary antibody with a FITC-conjugated secondary antibody. *L. intracellularis* infects intestinal epithelial crypts preferably in small intestines, and the process is characterized by proliferation of immature enterocytes, loss of goblet cells and destruction of normal epithelial architecture. Green fluorescence detected in intestinal crypts matched with typical patterns of pathogenic *L. intracellularis* localization during intestinal epithelial cells infection (7) (Fig. A.1A). White arrows point to the *L. intracellularis* infected crypts, where we see bacteria in green. Note that on both A and B, additional fluorescent signals localized in subepithelial spaces are also observed. This background fluorescence may be natural fluorescence of the gut or it may be an artifact from the extended time for tissue processing during formalin-fixation. In addition to the detection of fluorescent bacteria, lack of goblet cells and loss of normal epithelial architecture is obvious, especially when infected crypts (see white arrows) are compared to the uninfected crypts on the left side of (A) and throughout (B). Uninfected crypts have goblet cells in abundance, dark oval spaces, and no fluorescent signals beyond background levels detected in epithelial cells. IHC analysis of ileum samples from non-infected, negative control animal, showed that intestinal epithelial histology is preserved, with a normal number of goblet cells and no proliferation of immature cells in the crypts. Also, there are no fluorescent signals detected in epithelial crypts (Fig.A.1B)

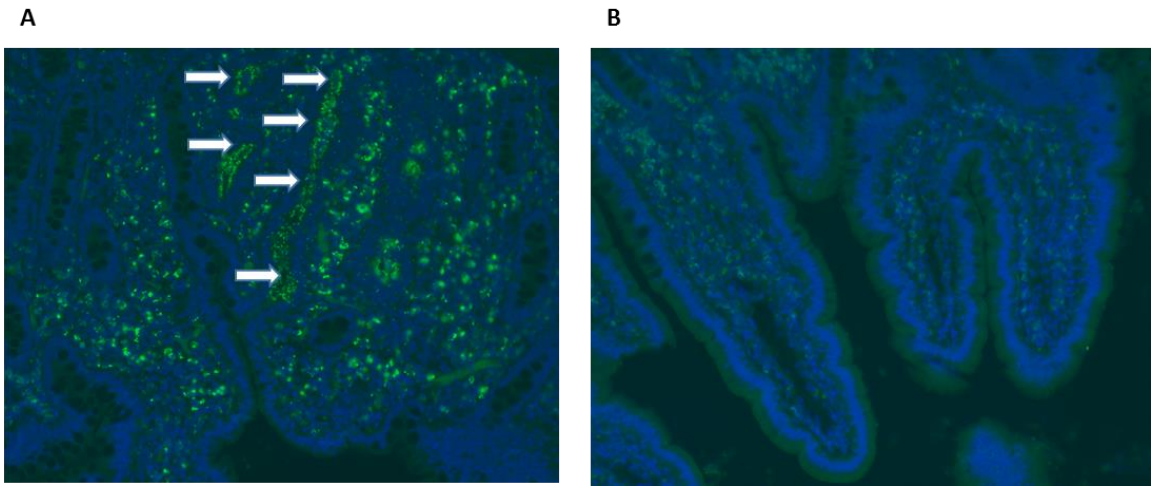

Figure A.1 IHC of the ileum of *L. intracellularis* infected piglet and uninfected pig. Blue fluorescence represents DAPI stained eukaryotic cell nuclei; green fluorescence represents fluorescent signal from the bound secondary FITC antibody; white arrows indicate the intestinal crypts infected by pathogenic bacteria. (A); intestinal mucosa from uninfected pig labeled with anti-*Lawsonia* primary antibody and FITC secondary antibody (B);

A.1.3.2 qPCR from Experiment 1

DNA extraction was performed on ileum samples processed for PCR analysis. TaqMan probe and 16S primers, explained above, were applied for detecting specific bacteria. The differences in this DNA extraction compared to DNA extraction from *Lawsonia* infected cell culture involved using a different buffer (250 mM NaOH, 2 mM EDTA) and in longer time heating on 95℃ to disrupt intestinal tissue cells. The Ct value of the sample from infected ileum was 25.0826; the Ct value of uninfected ileum from newborn piglet (negative control) was 36.7 and the Ct value of the uninfected ileum spiked with 10^5 avirulent *L. intracellualris* (positive control) was 21.41. Lower Ct values indicate higher quantity of *L. intracellularis* DNA which confirms infected tissue IHC positive status. In conclusion, we used IHC and PCR to confirm that the pathogenic bacteria from a *L. intracellularis* infected gilt remained infectious. .

A.1.3.3 Results from Experiment 2

Limited data on clinical signs received from the farm that experienced an outbreak of PE indicated that the gilt succumbed to the PHE in two days despite being previously vaccinated with Enterisol® vaccine.

Flow cytometry analysis was used to monitor infection of McCoy cells with pathogenic *L. intracellularis* isolated from the gut of infected animal received in 2018. For passaging, McCoy cells were grown in T-25 flasks. Non-infected McCoy cells, McCoy cells stained with mouse isotype primary and APC-conjugated secondary and McCoy cells stained with anti-*L. intracellularis* primary and APC-conjugated secondary antibody served as negative controls. Detection of *L. intracellularis* inside McCoy cells was achieved by methanol permeabilization of the cells and staining with specific primary antibody against outer membrane protein of *L. intracellularis* and secondary anti-mouse IgG2b conjugated with APC. The APC signal is detected in FL-4 channel in flow cytometry analysis. McCoy cells without bacteria were set as a negative control and gate B was determined (Fig. A.2A).

McCoy cells stained with anti-*L. intracellularis* primary and APC-conjugated secondary antibody showed 12.56% events in the FL-4 channel indicating that there was some nonspecific binding of the anti-*L. intracellularis* antibody to non-infected McCoy cells. McCoy cells infected with non-pathogenic *L. intracellularis* were used as a positive control (Fig. A.2C) showed 89.29% events in the FL-4 channel in Gate B. These results indicate successful staining and invasion of avirulent bacteria in the first passage. Flow cytometric analysis indicated that pathogenic *L. intracellularis* isolated from the infected pig showed 43.78% events in the FL-4 channel (Fig. A.2D). When corrected for the non-specific binding of the primary antibody to McCoy cells (shown in Fig. A.2B), McCoy cells infected with non-pathogenic *L. intracellularis* had 76.73%

events in the FL-4 channel and McCoy cells infected with pathogenic *L. intracellularis* had 31.12% events in the FL-4 channel (Fig. A.2 C and D). The qPCR results showed that McCoy cells infected with vaccine strain had a Ct of 22.1; McCoy cells infected with *L. intracellularis* intestinal isolate had a Ct of 26.2 while uninfected McCoy cells had a Ct of 34.6, and thus confirming the presence of bacteria after propagation in McCoy cells.

McCoy cells infected with a non-pathogenic or pathogenic bacteria display similar density plot as non-infected cells and absence of dead cells in lower forward/side scatter (data not shown), which is expected due to non-cytopathogenic nature of *L. intracellularis* in *in vitro* cell cultures.

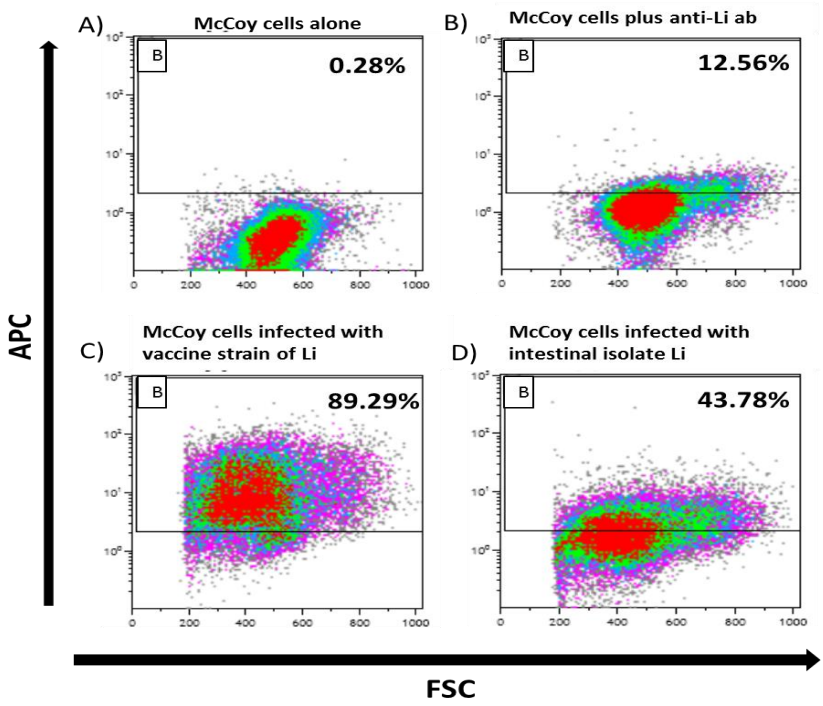

Figure A.2. Dot blots and gating for flow cytometry analysis of *L. inttracellularis* passage 1 in McCoy cells: A) gates on live McCoy cells forward scatter/APC plot, 0.28% of fluorescent events, B) McCoy cells stained with anti-*L. intracellularis* antibody and APC secondary antibody 12.56% of fluorescent events, C) McCoy cells infected with 10^4 vaccine strain *L. intracellularis* stained with anti-LI antibody and APC secondary antibody, 89.29% of fluorescent events, D) McCoy cells infected with intestinal isolate *L. intracellularis* stained with anti-*L. intracellularis* antibody and APC-conjugated secondary antibody, 43.78% of fluorescent events. Thirty thousand events were acquired for each sample.

Next, we wanted to determine if the bacteria continued to grow and replicate after other passages of McCoy cells. The McCoy cells were lysed and the bacterial pellet was acquired (explained in detail above). The bacterial pellet was suspended in DMEM + 7% FBS and 500 µl was used to infect each T-25 flask for passage number 2. To correlate infection with flow-cytometry, duplicate T-25 flasks were used wherein one flask was used for the next passage and the second flask was used for flow cytometry and PCR. Running two flasks allowed us to have the same environment for bacterial growth in each flask (the same number of McCoy cells, volume of medium, gas environment and days of bacteria growth) thus acquiring representative samples for bacterial growth monitoring analysis.

Fig. A.3 shows the flow cytometric analysis of the second passaging of non-pathogenic and pathogenic *L. intracellularis.* Cells and bacteria were processed as indicated above with the only difference being that the isotype control was added as an additional negative control (Fig. A.3A). Also, due to the presence of nonspecific binding of the anti-*L. intracellularis* antibody to McCoy cells observed in Fig. A.2, we included additional washes after staining.

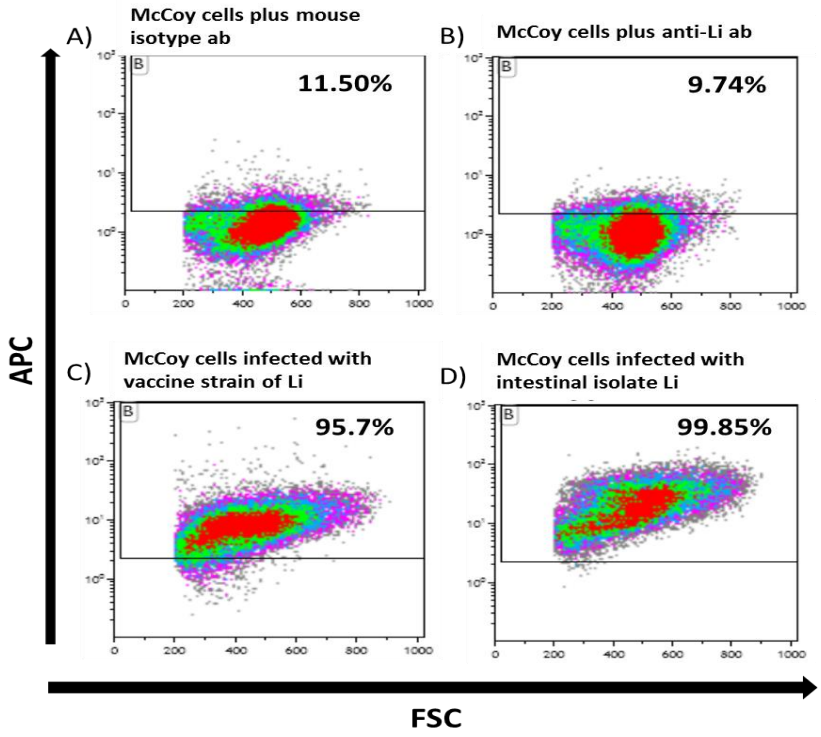

Figure A.3. Dot blots and gating for flow cytometry analysis of *L. intracellularis* **passage 2 in McCoy cells**: A) gates on live McCoy cells stained with anti-mouse isotype and APC-conjugated secondary antibody, forward scatter/APC plot, 11.50% of fluorescent events, B) McCoy cells stained with anti-LI antibody and APC secondary antibody 9.74% of fluorescent events, C) McCoy cells infected with 2nd passage vaccine strain *L. intracellularis* stained with anti-*L. intracellularis* antibody and APC-conjugated secondary antibody, 95.7% of fluorescent events, D) McCoy cells infected with 2nd passage intestinal isolate *L. intracellularis* stained with anti-*L. intracellularis* antibody and APC-conjugated secondary antibody, 95.85% of fluorescent events. Thirty thousand events were acquired for each sample.

Uninfected McCoy cells were used to set the gating parameters (data not shown). McCoy cells stained with mouse isotype antibody had 11.5% events in the FL-4 channel implying that indeed there is nonspecific binding of antibodies to this type of cell line (Fig. A.2A). Although we introduced additional washes after staining, non-specific binding of the anti-*L. intracellularis* antibody to non-infected McCoy cells was also observed in this passage with 9.74% events (Fig. A.3, B). Results from flow cytometry analysis of the second passage of vaccine and intestinal isolate *L. intracellularis* showed higher percentages of events in the FL-4 channel compared to the

flow cytometry analysis of the first passage. McCoy cells infected with non-pathogenic strain had 95.7% of events in the FL-4 channel (Fig. A.3C) and McCoy cells infected with *L. intracellularis* intestinal isolate had 99.85% events in the FL-4 channel (Fig. A.3D). When subtracted for the percentages of fluorescence events due to nonspecific binding, McCoy cells infected with second passage of non-pathogenic strain had 86.23% events and McCoy cells infected with second passage of intestinal isolate *L. intracellularis* had 90.11% events. This increase in fluorescent events in passage two compared to the first passage indicates that both strains were actively growing in cell culture and that bacteria were multiplying in passage two. These findings correspond to previous reports of *L. intracellularis* growth *in vitro* where bacteria was present in high infected cells (HIC) with HIC density of 80% after seven days of infection (Lawson et al 1993).

We repeated the passaging of *L. intracellularis* into McCoy cells for a third time. Our negative controls, McCoy cells stained with isotype and McCoy cells stained with anti-*L. intracellularis* antibody, had negligible fluorescence, 0.18% and 0.27% respectively (Fig A.3A, B), which indicates that additional washings between staining with primary and secondary antibody was efficient to reduce non-specific binding of primary antibodies to McCoy cells. Flow cytometry analysis of third passage showed that McCoy cells infected with second passage of *L. intracellularis* vaccine strain had 47.31% events and McCoy cells infected with second passage of *L. intracellularis* intestinal isolate had 36.6% of fluorescence events in the FL-4 channel (Fig. A.4 C and D).

Comparing the results of the third and second passage, we observed a decrease in fluorescence events in both strains indicating reduced growth of bacteria in this passage. We speculate that the use of Ziplock bags filled with the required tri-gas mixture was not suitably

maintained over time. Possible leaking of gases may have disturbed the gas environment necessary for bacterial growth. Recently, our lab acquired Tri-gas incubator which will allow us to have a controlled gas environment necessary for *L. intracellularis* growth for future experiments.

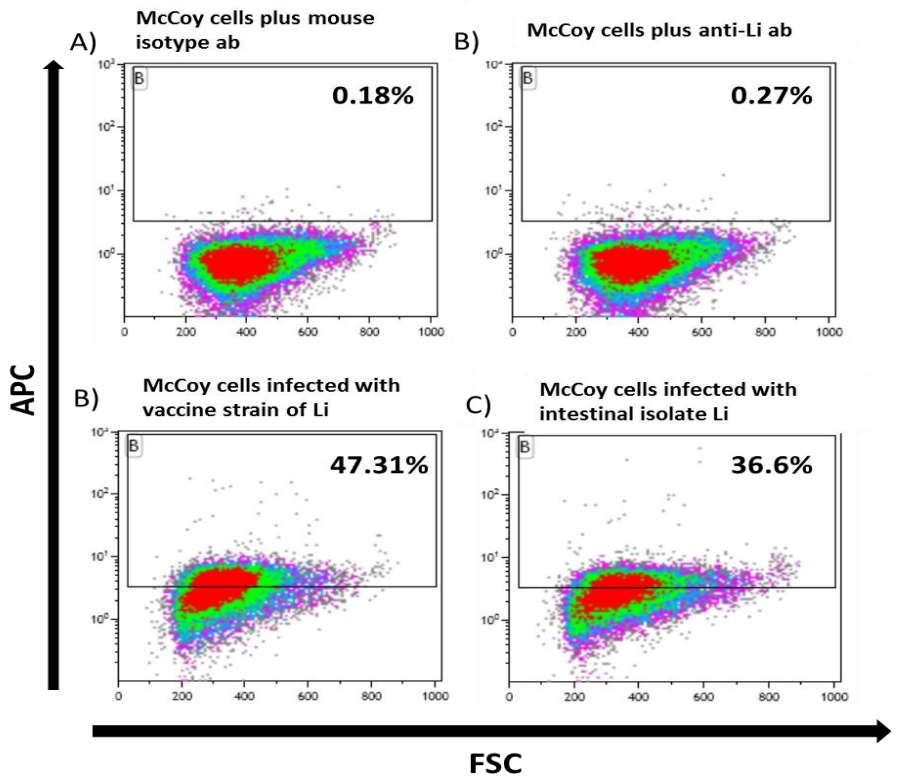

Figure A.4. Dot blots and gating for flow cytometry analysis of *L. intracellularis* **passage 3 in McCoy cells**: A) gates on live McCoy cells stained with anti-mouse isotype and APC secondary antibody, forward scatter/APC plot, 0.18% of fluorescent events, B) McCoy cells stained with anti- *L. intracellularis* antibody and APC secondary antibody 0.27% of fluorescent events, C) McCoy cells infected with 3rd passage vaccine strain *L. intracellularis* stained with anti- *L. intracellularis* antibody and APC secondary antibody, 47.31% of fluorescent events, D) McCoy cells infected with 3rd passage intestinal isolate *L. intracellularis* stained with anti- *L. intracellularis* antibody and APC secondary antibody, 36.6% of fluorescent events. Thirty thousand events were acquired for each sample.

A.1.4 Conclusion

In summary, we were able to process intestines from two cases of gilts diagnosed with PHE and used the intestinal homogenate to infect piglets. We were able to detect *L. intracellularis* in the intestine of infected piglets by using IHC and qPCR suggesting that the infection was successful. However, although a high number of bacteria were present in ileum crypts of piglets infected with *L. intracellularis* intestinal homogenate, we did not reproduce symptoms of PHE when it was used for infection. Clinical symptoms were mild with no diarrhea or melena. These findings may correspond to findings from the previous reports where younger animals have PIA form of disease while older animals, like gilts or breeding boars, tend to develop more acute and severe PHE form of the disease (6). As discussed previously in this thesis, the marked difference in clinical symptoms of PIA and PHE is related to the age of animal and maturation of the immune system and not due to genomic differences in bacterial isolates.

The *L. intracellularis* growth was monitored using flow cytometry and this method is accurate and efficient to estimate *L. intracellularis* infection, *in vitro*. Flow cytometry offers an unbiased analysis of the percentage of McCoy cells infected with *L. intracellularis*. We were able to follow infection for the three passages and to estimate the percentage of infected cells in each passage. In future, our lab will utilize the Tri-gas incubator to increase the number of successful passages of *L. intracellularis* intestinal isolate *in vitro* and to monitor passaging of bacteria with flow cytometry.

Student contribution: M.O. designed and performed all the experiments here-in, and wrote the Appendix.

A.2 Contributions to peer review articles

A.2.1 "Evidence for a common mucosal immune system in the pig"

Heather L. Wilson and Milan R. Obradovic

(As published in the Molecular Immunology, 2015; July, 66 (1): 22-34.)

Abstract:

The majority of lymphocytes activated at mucosal sites receive instructions to home back to the local mucosa, but a portion also seed distal mucosa sites. By seeding distal sites with antigen-specific effector or memory lymphocytes, the foundation is laid for the animal's mucosal immune system to respond with a secondary response should to this antigen be encountered at this site in the future. The common mucosal immune system has been studied quite extensively in rodent models but less so in large animal models such as the pig. Reasons for this paucity of reported induction of the common mucosal immune system in this species may be that distal mucosal sites were examined but no induction was observed and therefore it was not reported. However, we suspect that the majority of investigators simply did not sample distal mucosal sites and therefore there is little evidence of immune response induction in the literature. It is our hope that more pig immunologists and infectious disease experts who perform mucosal immunizations or inoculations on pigs will sample distal mucosal sites and report their findings, whether results are positive or negative. In this review, we highlight papers that show that immunization/inoculation using one route triggers mucosal immune system induction locally, systemically, and within at least one distal mucosal site. Only by understanding whether immunizations at one site triggers immunity throughout the common mucosal immune system can we rationally develop vaccines for the pig, and through these works we can gather evidence about the mucosal immune system that may be extrapolated to other livestock species or humans.

Dr. Wilson and I collected and critically evaluated the published data for evidence for a common mucosal immune system in pigs.

A.2.2 "**Development of flow cytometry-based adherence assay for *Neisseria gonorrhoeae* using 5′-carboxyfluorosceinsuccidyl ester"**

Sidharath Dev Thakur, Milan Obradovic, Jo-Anne R. Dillon, Siew Hon Ng, Heather L. Wilson (BMC Microbiol. 2019 Mar 25;19(1):67. doi: 10.1186/s12866-019-1438-2)

Abstract
Background: *Neisseria gonorrhoeae* is an obligate human pathogen and its adherence to host cells is essential for its pathogenesis. Gonococcal adherence assays are based on the enumeration of bacteria attached to human cells on solid media. Because conventional adherence assays are based on bacterial counts, they are often time consuming to perform and prone to observer bias. A flow cytometry based method, using the cell-permeable fluorescent dye 5′-carboxyfluoroscein succidyl ester (CFSE), was developed to dramatically increase the number of adherent *N. gonorrhoeae* quantified per assay while improving repeatability and removing observer bias.
Methods: Piliated *N. gonorrhoeae* F62were stained with CFSE then the staining reaction was quenched with foetal bovine serum. Human cervical ME-180 cells were infected with CFSE-stained *N. gonorrhoeae* (multiplicity of the infection 100:1) for 2 h. Infected cells were washed to remove loosely adhered bacteria. Flow cytometry was used to quantify the percentage of ME-180 cells associated with CFSE-stained *N. gonorrhoeae* and a minimum of 30,000 events were recorded. Real time-PCR analysis targeting *opa*gene (encoding *N. gonorrhoeae* opacity associated gonococcal outer membrane protein) was performed on infected ME-180 cells to confirm the flow cytometric adherence assay results. A rabbit was immunized with heat-killed *N. gonorrhoeae*F62 to generate hyperimmune serum. The functional compatibility of the assay was confirmed by studying the effect of *N. gonorrhoeae* F62 antiserum on blocking adherence/invasion of CFSE-stained bacteria to ME-180 cells
Results: We observed that 20.3% (+/- 1.0) ME-180 cells were associated with CFSE-stained *N. gonorrhoeae*. Heat-inactivated hyperimmune serum, at 1:10 to 1:80 dilutions, significantly inhibited gonococcal adherence by 6 and 3 fold, respectively. Real time-PCR analysis targeting *opa* gene confirmed that hyperimmune serum blocked adherence/invasion of *N. gonorrhoeae* to the ME-180 cells in a dilution-dependent manner.
Conclusions: Flow cytometric analysis was amenable to quick, easy and high-throughput quantification of the association of *N. gonorrhoeae* with ME-180 cells and was functionally confirmed using PCR analysis. These approaches may be adapted for *in vitro* and *in vivo* adherence studies related to gonococcal pathogenesis.

Dr. Sidharth Dev Thakur and I developed and performed neutralization assay and flow cytometry, PCR analysis, and cell culture analysis of *N. gonnorhea* in ME-180 cells.

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